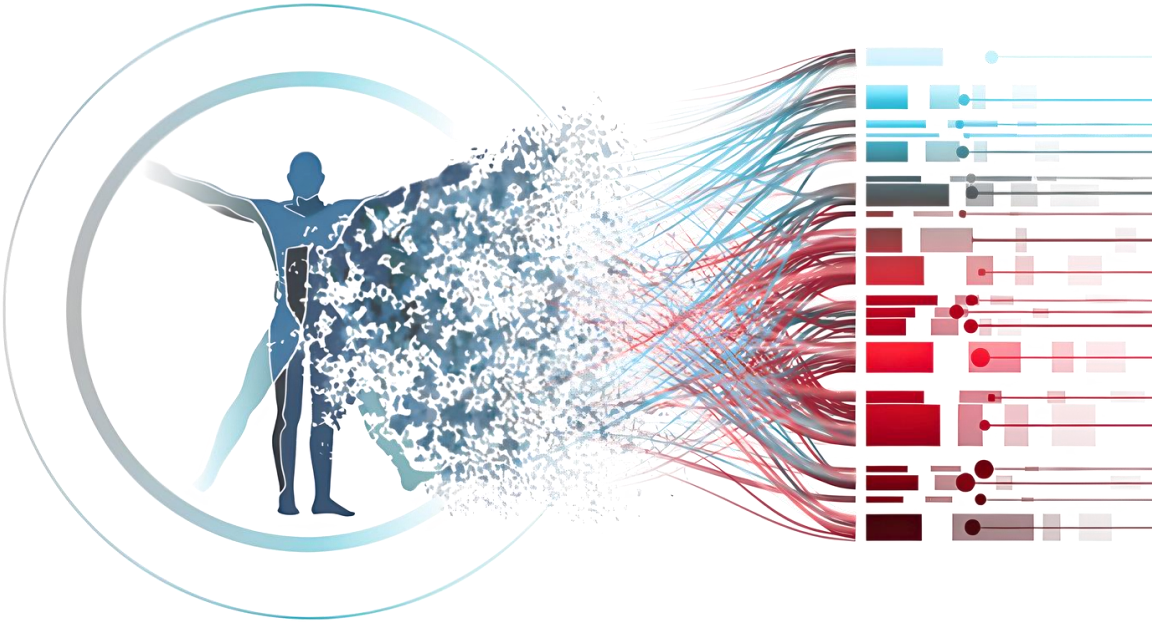
Bachelor's thesis

Ida Havikbotn

Addressing Analytical Challenges in Global Metabolomics: Application of Isotope-labeled Internal Standards for Quality Assurance

Analytiske Utfordringer i Global Metabolomikk: Bruk av Isotopmerkede Internstandardarder for Kvalitetssikring



Supervisor: Katja Benedikte Prestø Elgstøen
Co-supervisor: Eirik Sundby
May 2024

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Materials Science and Engineering

OSLO UNIVERSITY HOSPITAL

Addressing Analytical Challenges in Global Metabolomics: Application of Isotope-labeled Internal Standards for Quality Assurance

Analytiske Utfordringer i Global Metabolomikk: Bruk av Isotopmerkede Internstandarder for Kvalitetssikring

Ida Havikbotn

Bachelor Thesis in Chemical Engineering

20 credit points

Department of Materials Science and Engineering

Faculty of Natural Sciences

Norwegian University of Science and Technology

May 2024

2024

Addressing Analytical Challenges in Global Metabolomics: Application of Isotope-labeled Internal Standards for Quality Assurance

Abstract

Global metabolomics is a comprehensive analytical approach designed to identify all metabolites in a biological sample. It is valuable in clinical diagnostics, however the inherent non-specificity of the method poses challenges in quality assurance of test results. Particularly, discerning whether observed variations in metabolite levels between samples stem from genuine biological diversity or analytical issues presents a significant dilemma.

This thesis investigates whether the implementation of isotope-labeled internal standards (ILISs) could enhance the quality assurance in global metabolomics analyses. The study's primary objective is to facilitate an easier and more precise comparison of analysis results across diverse biological samples. Appropriate ILISs tailored to the metabolomic analysis conducted were carefully selected. Following optimization of concentrations in EDTA plasma, the ILIS solution was spiked into patient samples of clinically relevant biological matrices. Subsequent analysis, using liquid-chromatography mass spectrometry with electrospray ionization (LC-ESI-MS), assessed the potential of these standards as a quality assurance tool, comparing them against existing methods.

Through repeatable ILIS signals when spiking samples with 5.0 μM and 0.5 μM ILIS solution, the ILISs role as a quality assurance tool was confirmed. The ability to serve as quality assurance for individual samples was demonstrated – an ability that has not been possible before using existing quality assurance methods in global metabolomics. This not only enables more rapid interpretation of analysis results but also introduces a new era of precision and confidence in clinical diagnostics.

Sammendrag

Global metabolomikk er en omfattende analytisk tilnærming som er designet for å identifisere alle metabolitter i en biologiske prøve. Metoden er verdifull i klinisk diagnostikk, men den manglende spesifisiteten er en utfordring ved kvalitetssikring av testresultater. Det er spesielt vanskelig å bekrefte om observerte variasjoner i endogene signaler skyldes ekte biologiske variasjoner, eller analytiske problemer.

Denne studien undersøker om isotopmerkede internstandarder (ILIS) kan brukes for å forbedre kvalitetssikringen i globale analyser. Målet er at sammenligningen av analyseresultater mellom ulike biologiske prøver skal bli enklere og mer nøyaktig. Passende isotopmerkede internstandarder, spesifikke for den metabolske analysen utført, ble valgt. Etter optimalisering av konsentrasjoner i EDTA-plasma, ble løsningen av ulike ILIS'er tilsatt prøver av ulikt biologisk materiale. Væskekromatografi-massespektrometri med elektrospray-ionisering (LC-ESI-MS) ble brukt for å vurdere potensialet til standardene som et kvalitetssikringsverktøy, samt sammenligne de med eksisterende metoder for kvalitetssikring.

Gjennom repeterbare signaler for de isotopmerkede internstandardene ved tilsetning av 5.0 μM og 0.5 μM ILIS-løsning til biologiske prøver, ble det bekreftet at de isotopmerkede internstandardene kunne brukes som et kvalitetssikringsverktøy i global metabolomikk. Videre ble det også demonstrert at standardene kunne brukes som kvalitetssikring for individuelle prøver – noe som ikke har vært mulig med eksisterende kvalitetssikringsmetoder tidligere.

Preface

This study has been performed at the Department of Medical Biochemistry at Oslo University Hospital (OUS), Rikshospitalet from January 2024 to May 2024. My supervisors have been Katja B. P. Elgstøen at Rikshospitalet, and Eirik Sundby at the Department of Material Science and Engineering at Norwegian University of Science and Technology (NTNU).

I would like to express my gratitude to Katja for offering me the opportunity to work within her talented research group. It has been an incredibly experience to tackle clinical analytical issues that is relevant for future studies. Thank you for the unwavering encouragement, support, and supervision – it has been amazing to learn from the best!

Thank you to my supervisor in Trondheim, Eirik, for all the guidance through this process, always just a Teams call away.

A huge thank you to Sander for endless help and support throughout the project – from teaching me the basics, to discussing the tougher questions. Thank you for all the talks in the lab! It has been invaluable to always have a door to knock on to ask "silly" questions.

To the rest of the research group – thank you for bringing smiles to the department every day and for showing genuine interest in my work. A special thanks to Elise for all the willingness to answer my questions and lend a hand; it was truly appreciated.

Nathalie, thank you for being my partner in crime, and in the lab. I am very thankful for all our discussions, frustration, laughter, and companionship - we´ve been labelled the lab's "old married couple" for a reason. Thanks for sticking with me!

Lastly, a huge thank you to my boyfriend and family for cheering me on and being so patient during those crazy stressful times. Your support and encouragement have been a constant source of strength and motivation – thank you!

Ida Havikbotn

Table of Contents

Abbreviations

15:0-18:1 (d7) DG	1-pentadecanoyl-2-(9Z-octadecenoyl (d7))-sn-glycerol
15:0-18:1 (d7) PC	1-pentadecanoyl-2-(9Z-octadecanoyl (d7))-glycero-3-phosphocholine
15:0-18:1 (d7) PE	1-pentadecanoyl-2-(9Z-octadecenoyl (d7))-glycero-3 phosphoethanolamine
15:0-18:1 (d7) PG	1-pentadecanoyl-2-(9Z-octadecenoyl (d7))-glycero-3-phospho-(1'-sn-glycerol)
15:0-18:1 (d7) PI	1-pentadecanoyl-2-(9Z-octadecenoyl (d7))-glycero-3-phospho-(1'-myo-inositol)
15:0-18:1 (d7) PS	1-pentadecanoyl-2-(9Z-octadecenoyl (d7))-glycero-3-phosphoserine
15:0-18:1 (d7) TG	1-pentadecanoyl-2(9Z-octadecenoyl)-3-pentadecanoyl (d7)-sn-glycerol
18:1 (d7) Chol Ester	Cholest-5-en-3b-yl (9Z-octadecenoate (d7))
18:1 (d7) Lyso PC	1-(9Z-octadecenoyl (d7))-2-glycero-3-phosphocholine
18:1 (d7) Lyso PE	1-(9Z-octadecenoyl (d7))-2-glycero-3-phosphoethanolamine
18:1 (d7) MG	1-(9Z-octadecenoyl (d7))-glycerol
18:1 (d9) SM	N-(9Z-octadecenoyl (d9))-sphing-4-enine-1-phosphocholine
Ala/ Ala-d4	Alanine/ Alanine d4
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo-ionization
C15 Ceramide (d7)	N-(pentadecanoyl)-sphing-4-enine (d7)
C18-AC-d3	C18-Acylcarnitine/ C18-Acylcarnitiee-d3
CV	Coefficient of variation
DBS	Dried blood spot
DDA	Data-dependent acquisition
DIA	Data-independent acquisition

E	Mass accuracy
ESI	Electrospray ionization
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
HMDB	The Human Metabolome Database
HPLC	High-performance liquid chromatography
ILIS	Isotope-labeled internal standard
LC	Liquid chromatography
LOD	Limit of detection
m/z	Mass-to-charge
MeOH	Methanol
MS	Mass spectrometry
N.D.	Not detected
NMR	Nuclear magnetic resonance
NPLC	Normal phase liquid chromatography
OT	Orbitrap
PQC	Pooled quality control
PCA	Principal components analysis
QA	Quality assurance
QC	Quality control
R	Mass resolution
REK	Regional committees for medical and health research ethics

RPLC	Reverse phase liquid chromatography
RSD	Relative standard deviation
RT	Retention time
SD	Standard deviation
SST	System suitability test
TIC	Total ion count
TOF	Time of flight
Trp/ Trp-d5	Tryptophan/ Tryptophan d5
U/ U-$^{15}N_2$	Uracil/ Uracil $^{15}N_2$

1. Introduction

1.1. Clinical Diagnostics and Personalized Medicine

Clinical diagnostics is the process of identifying the nature of a disease or injury, and distinguishing it from other potential disorders (1). To make a prognosis and create a treatment plan, the diagnostic process may involve physical examination, laboratory testing, imaging tests, endoscopy, and biopsy, followed by medical reasoning (2). Uncertainty and time are two major complexities in this process. Consequently, medical technology continuously advances to minimize these challenges by developing sophisticated diagnostics tools, including metabolomic analytical platforms and informatics tools (3). Additionally, diseases that were previously thought to be distinct conditions can now be diagnosed thanks to these tools, and be further adapted to personalized medicine.

Personalized medicine refers to modification of treatments and diagnostics for specific patients depending on their individual physiologic and genetic traits (4). In contrast to the traditional evidence-based medicine, which typically employs a treatment-failure approach, personalized medicine can be utilized to predict, prevent and personalize treatment of diseases (5). Whilst genomics, transcriptomics and proteomics have been widely used for this purpose so far, metabolomics and lipidomics are being included to improve it even further.

1.1.1. Metabolomics – the final frontier of the "omics"

Metabolomics is a relatively recent addition to biomedical research, focusing on the comprehensive study of the metabolome, described in section 1.1.2 (6). Unlike genomics and transcriptomics, which provide information about potential biochemical statuses, and proteomics, which reveals available proteins, metabolomics offers insights into the dynamic metabolic state of the organism (7). This being said, each "omics" offers unique insights within clinical diagnostics, and represents the entire phenotype of an organisms, as illustrated in Figure 1 (5).

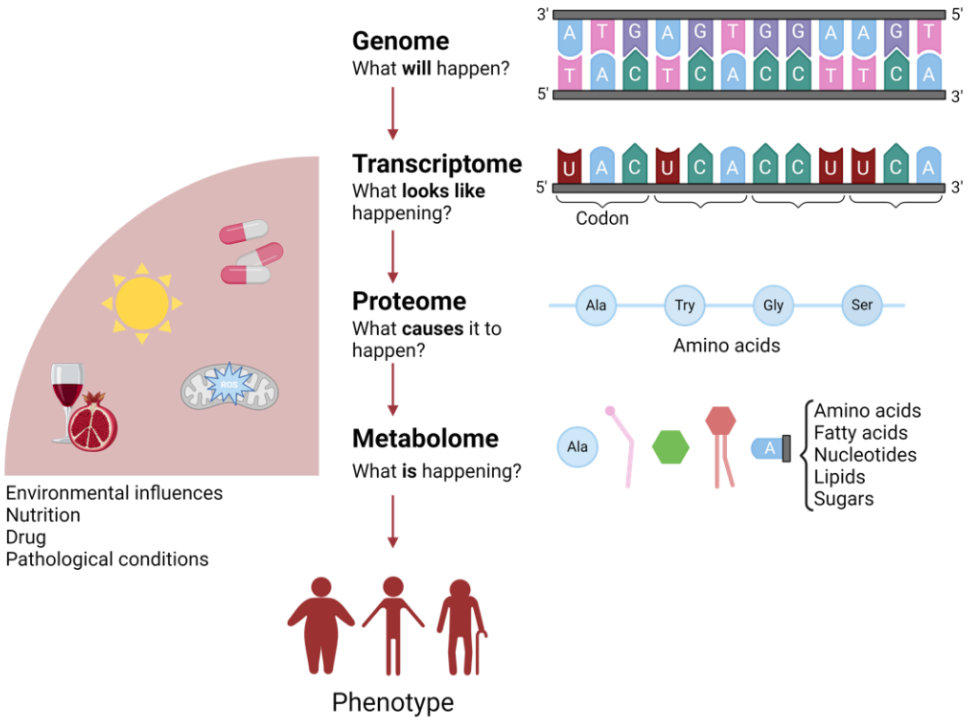


Figure 1 *The contribution of the four "omics" in the understanding of the phenotype. The genome predicts what will happen, the transcriptome describes what appears to be happening, the proteome identifies the factors causing it to happen, and the metabolome reveals what is happening. Additionally, some environmental factors influencing the transcriptome, proteome, and metabolome are depicted. Figure adapted from (8) using BioRender and Canva software.*

The metabolome, characterized by its inherently dynamic and flexible composition, continuously interacts within the biological system, and responds to external influences such as drugs, nutrition, lifestyle factors, and therapeutics (8). Changes in metabolite levels can serve as important indicators of complex diseases and monogenetic disorders, like for example inborn errors of metabolism. Metabolomics offers a means to measure and analyze these changes, capable of profiling a significantly broader range of metabolites than other diagnostic tools (5). This thorough coverage enables a deeper understanding of biological processes and pathways, highlighting the complex interplay between metabolites and physiological states. Therefore, any perturbations in metabolite levels serve as a true reflection of the phenotype and functional state of the biological system, whether in a developmental or pathological context (8).

Metabolomics consists of both targeted and global approaches (Figure 2). Targeted metabolomics involves the identification and quantification of a limited number of selected metabolites, typically requiring the use of multiple biological matrices to cover specific

metabolite classes (9). This approach offers the advantage of precise and sensitive quantification but relies on prior knowledge of the chemical and physical properties of the metabolites of interest. Analyzing multiple metabolite classes using targeted methods can be time-consuming due to the need to individually prepare samples for each targeted metabolite class. Furthermore, this method provides information solely about the selected metabolites, overlooking the vast array of other metabolites present in the metabolome.

In contrast, global metabolomics can be less time-intensive as it does not involve the selection of specific metabolites and it does not need multiple biological matrices (9). However, the interpretation of results after analysis can be very time consuming, and sometimes impossible. The objective of this approach is to comprehensively identify all metabolites present in a biological sample, regardless of their chemical properties. To achieve this, effective analytical platforms, bioinformatic tools and software are essential. One advantage to this approach is its ability to provide an advanced view of the body's pathophysiological state, making it valuable in clinical diagnostics. One notable application entails the comparative analysis of metabolites between control and test groups to distinguish between healthy and sick individuals.

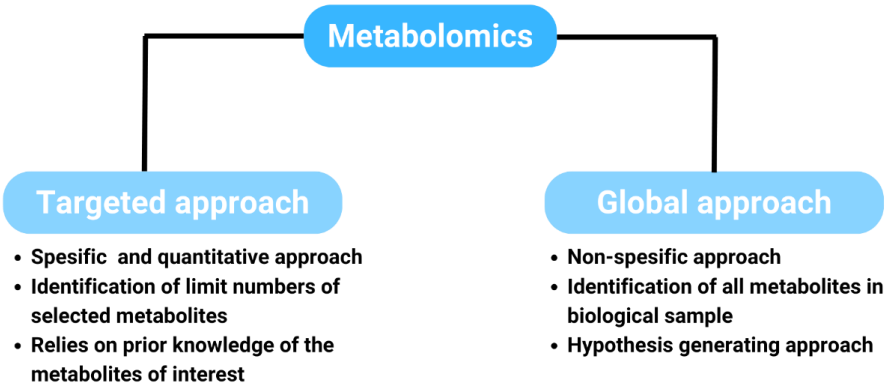


Figure 2 *Metabolomic methods – targeted and global approach. Figure was adapted from (9) using Canva software.*

The global approach not only saves time and resources but also enables a more comprehensive analysis of the metabolome.

1.1.2. Metabolites and the Metabolome

Understanding the role of metabolites and their interactions within biological systems is essential in biomedical research. Metabolites, characterized by their low molecular weight

(<1.5 kDa), encompass two main categories: endogenous and exogenous metabolites (7). While exogenous metabolites originate from the external environment, such as diet or pharmaceuticals, endogenous metabolites are products resulting from genomic coding. Serving as substrates, intermediates, or end products of enzymatic reactions, metabolites encompass a diverse array including amino acids, lipids, organic acids, and carbohydrates. Metabolism is the synthesis or utilization of energy and essential materials crucial for growth, reproduction, and overall health maintenance (10). The total amount of metabolites within a biological object is referred to as the object´s metabolome, and the study of it holds significant value in biomedical research as it reflects the interactions between the genome, transcriptome and proteome (7). For instance, exploring the human metabolome provides insights into pathophysiological mechanisms and facilitates the identification of novel diagnostic.

The human metabolome contains an extensive range of metabolites. The world's largest metabolomic database, "The Human Metabolome Database" (HMDB) 5.0, has identified over 220 000 metabolites of various chemical natures (11). The diversity among these metabolites is significant, as shown in Figure 3. The most substantial portion of the metabolome is lipid and lipid-like compounds, while the non-lipid compounds make up a far smaller percentage. However, they do exhibit a much broader range of classes than the lipid-soluble fraction.

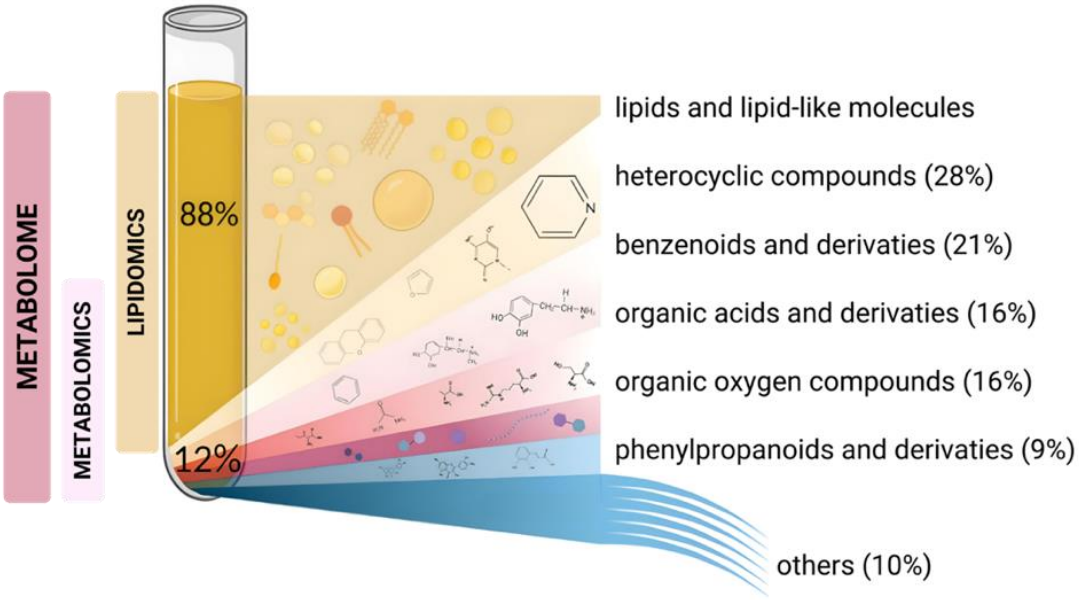


Figure 3 *Diversity of human blood metabolites, based on "The Human Metabolome Database" (HMDB) 5.0. Lipids constitute most of the metabolome. Remaining portion encompasses various classes such as heterocyclic compounds, organic acids, phenylpropanoids, and more. Figure adapted from (7) using Canva software.*

1.1.3. Biological Matrices for Metabolomics

Metabolites are widely distributed across various biological matrices, allowing metabolomics analyses to be conducted in diverse samples including urine, tissue, and other biological materials (7). Among these matrices, blood emerges as a key medium for probing the human metabolome. This preference arises from its easy extraction methods and analytical convenience. Blood provides a dynamic and comprehensive "snapshot" of the body's metabolic activity, reflecting the transformations of substances and energy occurring in all organs and tissues.

Whole blood can be separated into plasma and serum, each with distinct advantages for metabolomic analyses. Plasma closely resembles circulating blood after centrifugation, and it is obtained by preventing coagulation with anticoagulants like EDTA or heparin. Plasma is preferred in some studies due to its more reproducible and rapid processing, without the need to wait for blood clotting, which can vary among individuals. On the other hand, serum is the fluid obtained from coagulated whole blood, lacking fibrinogen, a crucial protein involved in clotting. It is considered a more “metabolite-rich” matrix with generally higher concentrations of most metabolites compared to plasma. However, serum may undergo amino acid conversions during clotting at room temperature, which could affect certain metabolomic profiles.

Importantly, in metabolomic analyses, comparisons are typically made between plasma samples, and between serum samples to ensure consistency and reliability in data interpretation. Despite the differences, both plasma and serum offer comparable analytical opportunities, and the choice between them often depends on specific experimental requirements.

Dried blood spot (DBS) samples offer an alternative sample collection method for capillary blood. It is collected by placing a drop of blood on a piece of filter paper, then dried (12). Despite the straightforward nature, DBS samples provide a comparable coverage of metabolites to plasma/serum samples (9). They are valued for their simplicity and long-term stability, making them valuable in clinical diagnostics, such as in newborn screening in Norway. They can also be transported without the need for storage on ice, and can be stored for prolonged periods (12).

Overall, the dynamic nature of blood enhances its utility in metabolome analysis. Considering

the metabolome's diverse components with varying physical and chemical characteristics, a comprehensive analytical platform is essential in global metabolomics (7).

1.2. Analytical Techniques used in Global Metabolomics

As the human body contains an extensive number of diverse metabolites, there is not a single analytical platform to analyze the entire metabolome (6, 13). In global metabolomics, the analytical platforms nuclear magnetic resonance (NMR) and mass spectrometry (MS) are commonly used, each with advantages and disadvantages.

NMR exploits the resonance of protons within a strong magnetic field, facilitated by a powerful magnet to precisely align protons within the sample. This technique offers the potential for quantification through internal standards and enables non-destructive detection of a broad range of metabolites simultaneously. It is particularly effective for detecting known metabolites up to micromolar levels, as illustrated in Figure 4. NMR also yields highly reproducible results that do not require signal correction, thereby simplifying data interpretation across experiments (6).

In contrast, MS measures mass-to-charge (*m/z*) ratios of metabolites, providing better sensitivity, especially in detecting metabolites at low picomolar levels. This makes MS well-suited for uncovering unknown metabolites, and is the reason why it is the predominantly favored technique in metabolomic analysis. Additionally, MS offers the advantage of easy connection with separation instruments such as liquid chromatography (LC) or gas chromatography (GC) (12). In global metabolomics, LC is often preferred over GC for its versatility. GC is limited to volatile compounds, which are less common among metabolites in living organisms. Additionally, it uses high temperatures which is a disadvantage when dealing with unstable metabolites (7).

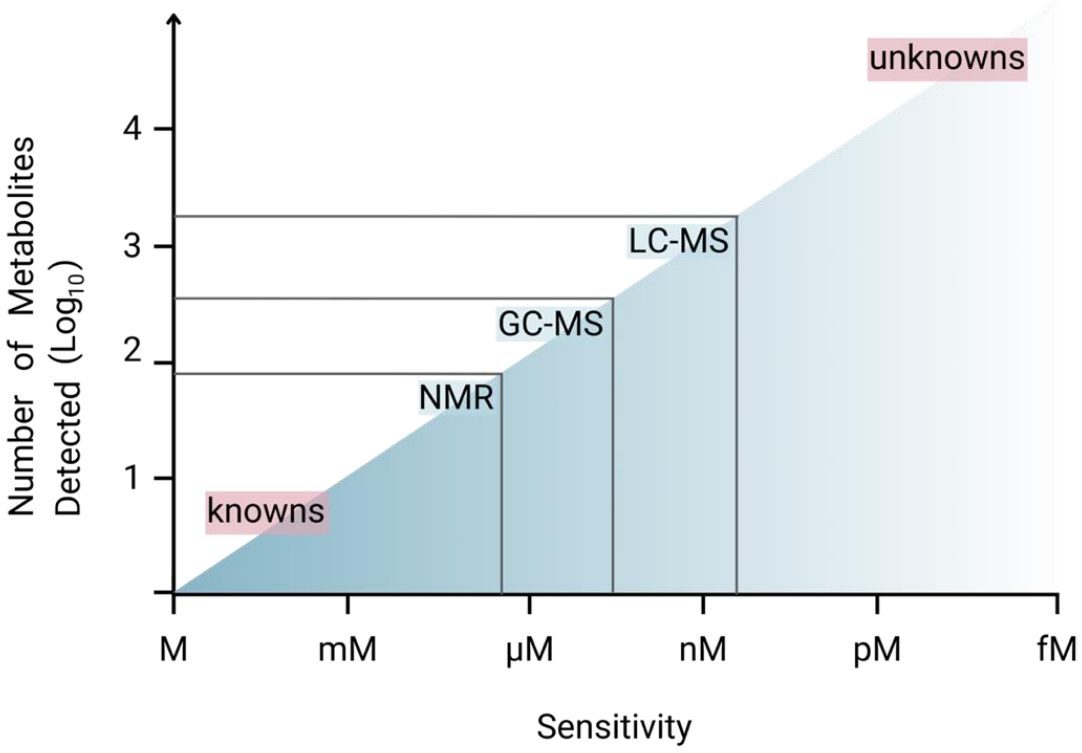


Figure 4 *Comparison of nuclear magnetic resonance (NMR), liquid chromatography-mass spectrometry (LC-MS), and gas chromatography-mass spectrometry (GC-MS) sensitivity and metabolite detection. Figure adapted from (13) using Canva software.*

1.2.1. Mass Spectrometry

Mass spectrometry in metabolomics relies on the detection and characterization of metabolites by their *m/z* ratio and fragmentation spectra (MSMS) (6). Firstly, a sample is introduced through a sample inlet into the instrument. An ion source transitions the analytes from a liquid phase to gas phase. Subsequently, the ions are directed into a mass analyzer where they are separated according to their mass-to-charge ratio. Upon exiting the mass analyzer, the ions are detected by a detector which produces an electric current proportional to their abundances. To minimize ion loss, both the mass analyzer and detector are maintained under vacuum conditions, as is the ion source occasionally. Finally, the recorded data is processed by a dedicated data system. The components are illustrated in Figure 5, and further explained in the following paragraphs.

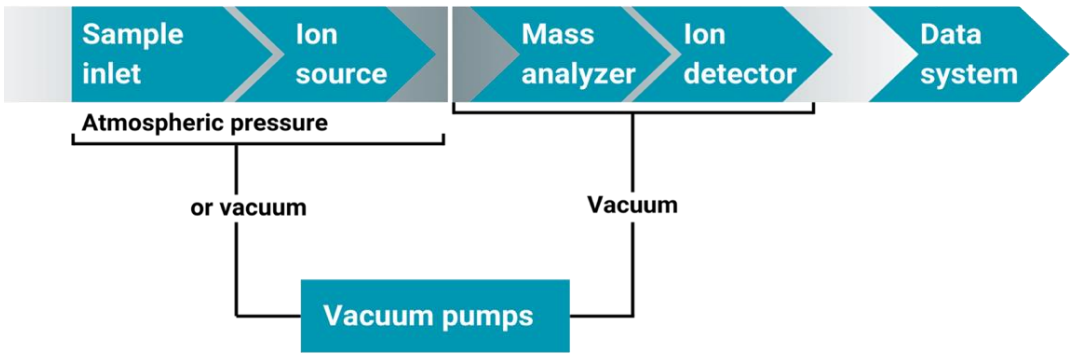


Figure 5 *Schematic representation of the components in a mass spectrometry system. The mass analyzer and ion detector require vacuum from the vacuum pumps to maintain optimal conditions. Additionally, some sample inlets and ion sources may operate under atmospheric pressure or vacuum, depending on specific experimental requirements. Figure made using Canva software.*

Ion Source

The ionization of metabolites occurs at the interface between the LC and the MS, facilitated by an ion source (12). Common ion sources in metabolomics include electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), and atmospheric pressure photo-ionization (APPI). Among these, ESI is the most popular method in metabolomics due to its effectiveness in ionizing a wide range of metabolites spanning various polarities. The scope of polarity and molecular weight covered by different ion sources is depicted in Figure 6.

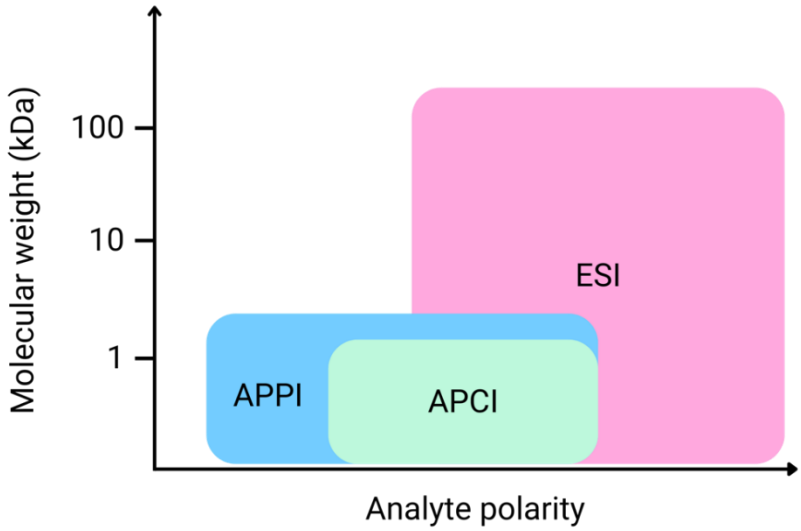


Figure 6 *The coverage of polarity and molecular weight by key ion sources used in metabolomics: Electrospray ionization (ESI), atmospheric pressure photo-ionization (APPI), and atmospheric pressure chemical ionization (APCI). ESI, with its broad versatility, occupies the largest area. Figure adapted from (12) using Canva software.*

ESI is considered a soft ionization technique, producing ions with minimal uncontrolled fragmentation, and rarely requiring sample derivatization (12). In this process (depicted in Figure 7), the sample carried by the mobile phase enters a capillary where a high voltage (typically ±5 kV) is applied. This voltage generates a Taylor cone – a jet of charged particles – that mixes with a nebulizing gas (usually N_2) at the end of the capillary, forming highly charged droplets. As these droplets travel toward the mass analyzer, they decrease in size due to mobile phase evaporation, leading to increased charge repulsion within the droplet. When these repulsive forces exceed surface tension, the droplet explodes (Coulombic explosion), resulting in smaller droplets. The charged ions then enter the mass analyzer due to the vacuum inside the MS.

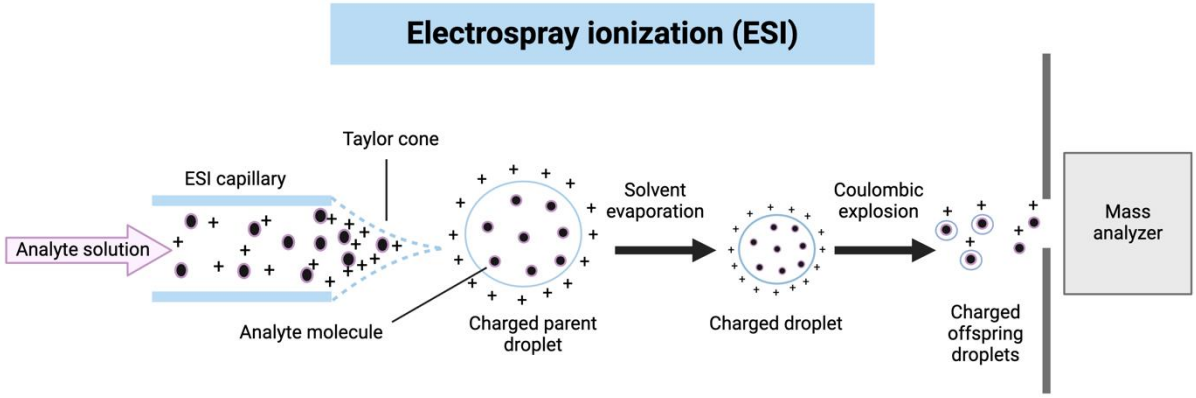


Figure 7 *Schematic representation showing the process of electrospray ionization (ESI). The analyte enters a capillary where high voltage leads to the formation of a Taylor cone. When mixed with a nebulizing gas, charged droplets are formed. When moving towards the mass spectrometry (MS) the droplets decrease in size while the charge increases before it explodes (Coulombic explosion). Charged ions then enters the mass analyzer. Figure is adapted from (12) using BioRender software.*

Mass Analyzer

The mass analyzer separates the ions from each other by their *m/z* value (12). Its performance can vary depending on the type of analyzer, and is described by mass resolution, mass accuracy, and scan speed. The mass resolution (R) says something about the ability to separate *m/z* values from each other, while the mass accuracy (E) describes the difference between measured *m/z* values and theoretical *m/z* values. A high R value and a low E value is desired. Scan speed says something about the time needed to obtain the mass spectrum, and should be 12-15 data points per peak. The performance of the mass analyzer is critical in global metabolomics due to the essential need for high resolution.

There are various mass analyzers used in mass spectrometry, with two common ones being time-of-flight (TOF) and orbitrap (OT). The TOF analyzer accelerates ions in an electrical field between two plates (pulsed source), measuring the time of flight from the beginning to the end of a flight tube. Lighter ions reach the detector faster than heavier ions, and the *m/z* value is determined from the measured kinetic energy. In contrast, the orbitrap analyzer employs a different approach, trapping ions around a central electrode and allowing them to oscillate along the axis of the analyzer (6). This oscillation, induced by an electric field between two outer electrodes, yields a frequency directly proportional to the ions' *m/z* ratio, determined through the process called Fourier Transformation. OT offers good mass accuracy and high mass resolution, and is widely used in metabolomics (7, 14).

Mass analyzers can also be combined to enable ion fragmentation and obtain fragmentation spectra (MSMS) (12). This combination, known as tandem MS, is illustrated in Figure 8. The first mass analyzer (MS1) selects specific ions based on their *m/z* values to send to the collision cell, where they undergo fragmentation, and the resulting fragments are measured (MS2).

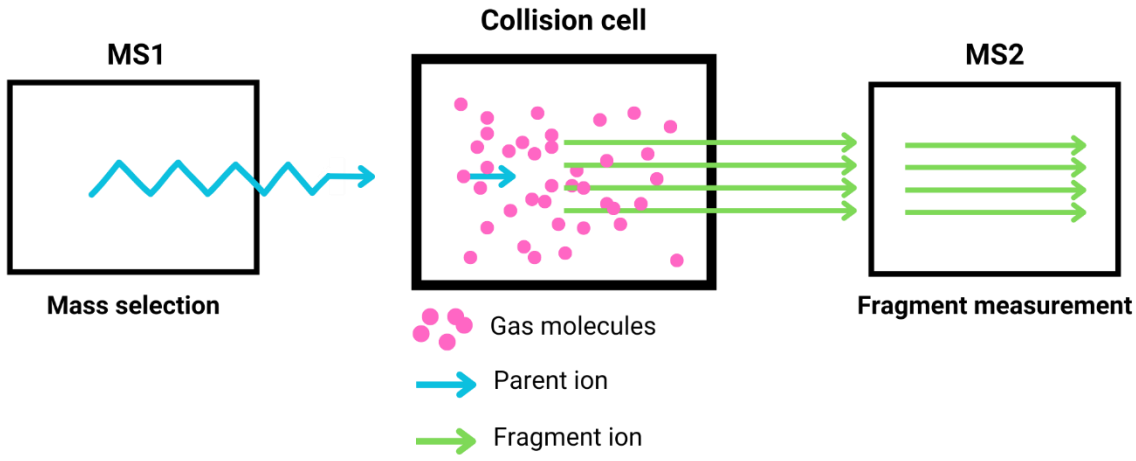


Figure 8 *Tandem MS where a parent ion from the first mass analyzer (MS1) is sent to a collision cell where it is fragmented. The fragmentation of the ion is then measured (MS2) to obtain a fragmentation spectrum for this particular ion. Figure adapted from (12) using Canva software.*

Once the analytes exit the mass analyzer, they are subjected to detection by the detector, provided they surpass the limit of detection (LOD) (12). The LOD represents the minimum signal intensity required for reliable detection of analytes with a certain probability, as determined by the peak-to-peak noise level.

1.2.2. High-Performance Liquid Chromatography

Given the diversity of compounds in bioanalysis, a separation method is often used (12). High-performance liquid chromatography (HPLC) serves this purpose by separating compounds according to their distribution between two distinct phases – the stationary phase and the mobile phase, the latter being a liquid as per the liquid chromatography method (15). Employing high pressure generated by a pump the mobile phase – responsible for sample movement – is driven through enclosed columns containing the stationary phase (16). Compounds exhibiting strong affinities to the stationary phase stay within the column for extended times, whereas those with weaker affinities elute more rapidly. This binding affinity depends on the molecular structure and composition of each compound, resulting in distinct interactions and elution patterns. The interval between sample introduction and elution is termed as retention time (RT) (15).

HPLC is acknowledged for its ability to effectively separate a broad range of molecules, spanning molecular weights from 50 to several millions Da. Because of this capability, this method stands out as a premier choice for the separation of complex multicomponent mixtures, including those encountered in biological samples. The HPLC system is depicted in Figure 9.

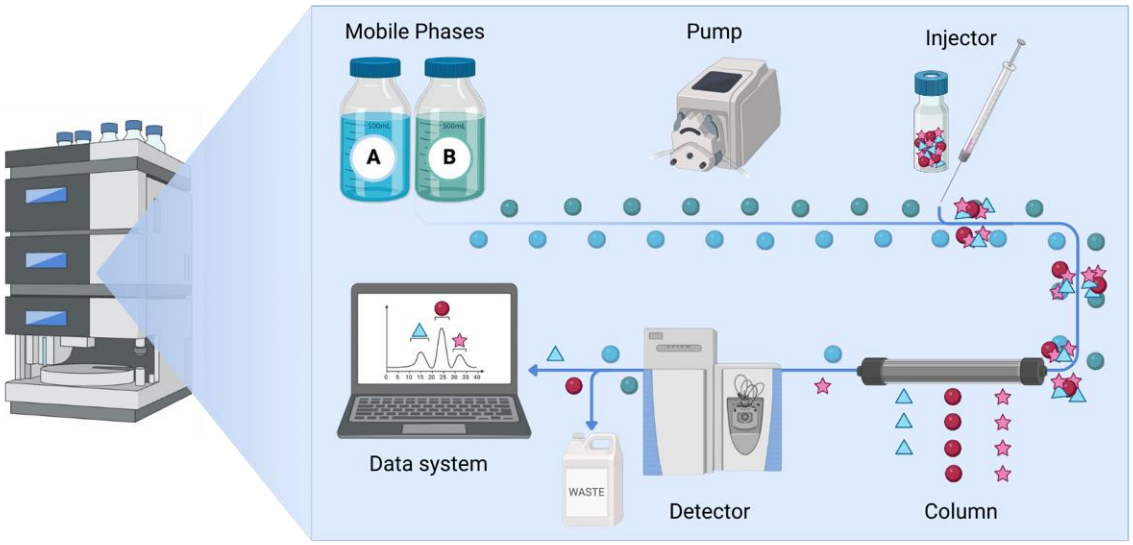


Figure 9 *The main components and steps of high-performance liquid chromatography (HPLC) analysis. A sample is introduced by the injector, moved by the mobile phase, and separated in the column before detected by a detector. Figure adapted from (12) using BioRender and Canva software.*

For metabolomics, both reverse phase liquid chromatography (RPLC) and hydrophilic interaction liquid chromatography (HILIC) can be employed (17). RPLC employs a hydrophobic stationary phase and a more polar mobile phase to separate semi-polar compounds like phenolic acids, glycosylated steroids, alkaloids, flavonoids, and other glycosylated species using a C18 column (12, 17). In contrast, normal phase liquid chromatography (NPLC) features a polar stationary phase and a less polar mobile phase. HILIC, a versatile type of NPLC, uses silica as the stationary phase and adjusts the polarity of the mobile phase. This technique is often used to separate relatively polar analytes such as sugars, vitamins, amino acids, nucleotides, carboxylic acids, and ionic substances not effectively separated by reversed-phase mode. While effective, NPLC is less commonly employed in metabolomics due to challenges in coupling it with ESI-MS. This is because ESI-MS works best with polar solvents, and NPLC uses non-polar organic solvents as the mobile phase.

1.3. Workflow in LC-MS Global Metabolomics

The global metabolomic workflow employing LC-MS exhibits considerable variability, yet involves several key stages, as depicted in Figure 10 (18).

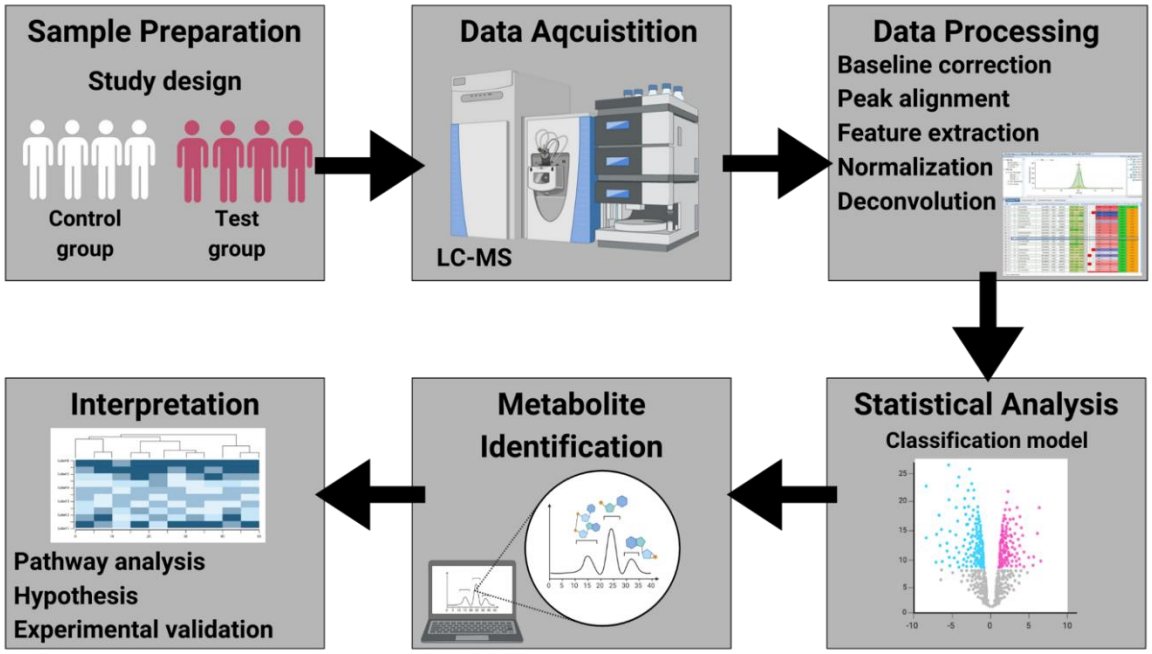


Figure 10 *The hypothesis generating workflow of global metabolomics. Biological samples are prepared before data acquisitioned. Subsequent steps involve processing the data, identifying metabolites within the dataset, and statistical analysis to extract meaningful insights. Based on these findings, a hypothesis is formulated, guiding subsequent experimental validation to either confirm or disprove the hypothesis. Figure is adapted from (18, 19) using BioRender and Canva software.*

Achieving analytical repeatability is crucial for the success of the experiment, as it ensures that the data accurately reflects biological variations. Hence, this also reduces the need for numerous replicates. Coupled with data analysis, the primary goal is to derive a hypothesis from the findings, followed by experimental validation, such as targeted analyses, to either validate or disprove the hypothesis.

1.3.1. Data Acquisition

During data acquisition in LC-MS global metabolomics, various scanning modes can be utilized to capture the metabolome (20). Full scan mode is primarily employed for metabolite detection, while MSMS is used as a confirmation of the identity, as described in part 1.2.1. This mode also facilitates semi-quantification by comparing signal intensities between patient and control groups.

There are alternative MSMS acquisition methods which can be used, such as data-independent acquisition (DIA) and data-dependent acquisition (DDA). DDA is the predominant mode in global metabolomics. It automatically transitions from full scan mode to MSMS when precursor ions meet predefined criteria, typically targeting a set number of precursor ions with the highest intensities (commonly five) for fragmentation. In contrast, DIA allows the user to predefine fragmentation without dependence on precursor ion intensity.

Quality Assurance and Quality Control in Data Acquisition

Quality assurance (QA) and quality control (QC) are crucial for ensuring reliable data acquisition in metabolomics (21). QA covers pre-analysis procedures and efforts to meet quality standards and requirements, including activities such as system suitability testing (SST) and MS-calibration. On the other hand, QC involves specific measurements during instrumental analysis, to ensure that quality requirements are met.

The system suitability test typically begins with a blank gradient run, which involves analyzing a solvent-only gradient to identify and eliminate impurities or column contamination. Following this, a solution containing chemical standards (5-10 analytes), dissolved in a suitable diluent, is analyzed. These analytes are used to evaluate the instrument´s performance under clean sample conditions, free from biological matrix effects.

Furthermore, it is essential that they cover a broad range of *m/z* values and retention times to comprehensively cover the entire analysis. The results are then evaluated against specific analytical criteria tailored to the requirements of the analysis, including *m/z* ratio and chromatographic characteristics such as retention time, peak area, and peak shape. Performing the SST allows for the early detection of potential changes in analytical instrumentation, before conducting the analysis of important biological samples. This enables corrective actions to prevent any impact on test results.

Pooled QC Samples for Quality Control

In global metabolomics, pooled samples commonly serve as QC to ensure reliability and repeatability (22). These samples, called pooled quality control (PQC) samples, are created by pooling aliquots from all samples in a study, thus representing the metabolites across the samples. By running them intermittently between test samples (typically every fifth sample or more) the variability in instrumental performance can be monitored to some extent.

Consistency in results is confirmed by assessing the performance of the PQC samples. If significant variation in signal strength is observed among PQC samples, a reanalysis of the entire set of patient samples may be necessary. Furthermore, if one PQC sample shows notably different signal strength compared to the others, sample injections around this quality control point must be excluded from further evaluation unless the cause is clear. Laboratories typically establish predefined limits for acceptable variation between PQC samples.

In addition to this, the PQC can be used as peak alignment to ensure stable retention times across samples (21). Ideally the injections of the PQC sample have identical signal intensities and retention times throughout the analysis run as they consist of the exact same metabolites with equal concentrations. However, real-world variations often deviate from this ideal scenario, thus software tools are used to detect the changes in measured *m/z* values within the PQC samples. These changes are then used to adjust the signal intensities of each *m/z* value in all test samples, aligning them with the corresponding alterations observed in the PQC signals within the same timeframe. This adjustment helps enhance the precision and reliability of metabolomic data analysis.

1.3.2. Data Processing

Following data acquisition, data processing is necessary to eliminate systematic bias occurring from the instrumental analysis to find and identify differences between samples (23). This process involves performing retention time alignment. Then, unknown compounds over a specified mass tolerance are detected and grouped across all samples by using algorithms. Gaps are also filled, and the chemical background are hidden using blank samples. These steps are often referred to as normalization and deconvolution. Identification of compounds is then done by using MS2 data, and online and inhouse libraries.

1.3.3. Statistical Analysis

Furthermore, statistical analysis are done to identify significant differences in metabolite abundance relationships, and it includes visualization tools to aid interpretation (23). Two main approaches are used – univariate and multivariate analysis. Univariate methods analyze metabolomics features individually, offering simplicity and straightforward interpretation (23, 24). It does not account for interactions between metabolomic features, such as pathway correlations and metadata, which can lead to increased risk of false positives/negatives. However, there are statistical tests that deal with these issues.

In contrast, multivariate methods analyze features simultaneously, capturing interactions and effectively detecting correlated pattern among biological variables. Principal components analysis (PCA) is a common multivariate method used to simplify complex datasets by identifying patterns and reducing the number of variables (25). These new properties capture most of the original data's signal, allowing for easy visualization and identification of patterns or groups within the data. Both methods are used in global metabolomics.

1.3.4. Metabolite Identification

Obtaining meaningful biological or scientific insights from a spectral peak relies on metabolite identification and confidence levels, as depicted in Figure 11 (26). Level 5 of confidence requires that a compound exhibits unique features with distinct *m/z* (± 5 ppm) and retention time. Level 4 of confidence adds predictive molecular formula based on the isotope distribution in the MS1 spectrum. At level 3, precursor *m/z* values match with entries in a metabolite database, whilst level 2 matches fragmentation data with MSMS libraries. Finally,

level 1, where MSMS spectrum and retention time of the feature match with analytical reference standard data, obtained by analyzing with the same conditions as the samples.

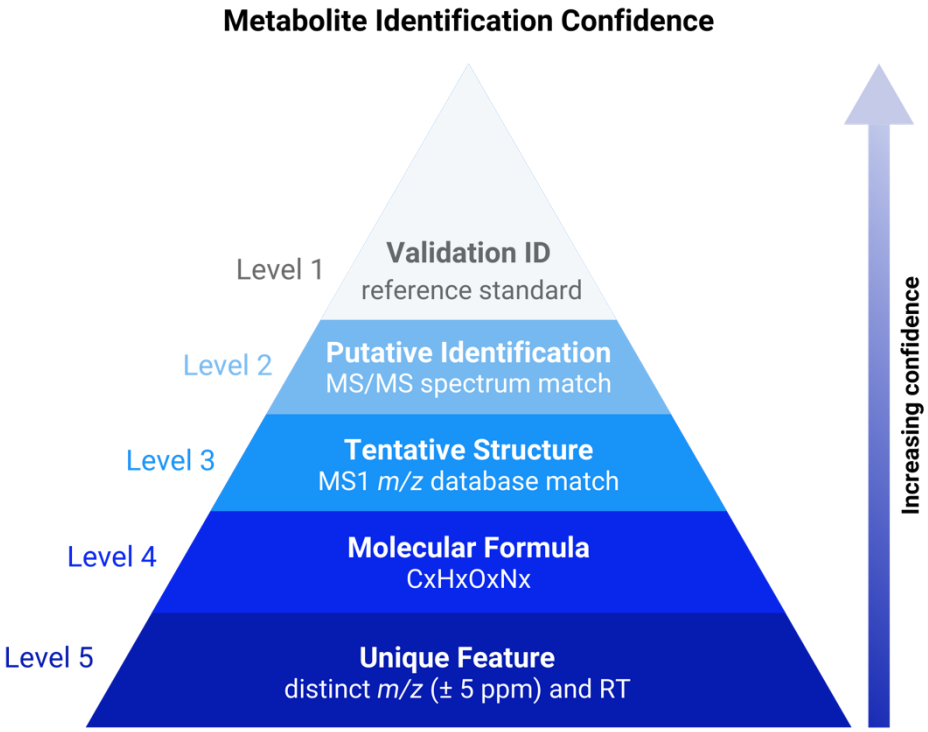


Figure 11 *The level of confidence triangle for metabolite identification. The confidence in identification of a metabolite is divided into five levels. Level 5 represents the lowest confidence, where only a unique feature (m/z or retention time (RT)) is observed. Level 1 corresponds to the highest confidence, indicating validated identification. Figure adapted from (26) using Canva software.*

Following statistical analysis and metabolite identification, biological interpretation involves further analysis to generate hypotheses and validate experimental data.

1.4. Challenges in Quality Assurance for Global Metabolomics

One of the major bottlenecks in global metabolomics remains the challenges in quality assurance of identifying metabolites in a biological sample (27). The diversity of analytical methods used in global metabolomics, complicates the development of a uniform quality assurance protocol (22). As highlighted in chapter 1.3.1, PQC are often used for quality assurance in this field, however, they do have limitations.

1.4.1. Limitations of PQC for Quality Assurance of Data and Test Answers

PQC samples serve as a primary tool for quality assurance in metabolomic analysis, however, they do have limitations (22). While PQCs can assess instrumental performance during analysis to some degree, they cannot ensure the quality of other critical factors such as sample preparation. This limitation arises because PQCs are not incorporated into actual study samples but are prepared and analyzed separately. Consequently, it is impossible to identify if specific test samples have issues between PQC assessments.

Furthermore, PQCs are unable to fully guarantee consistent instrumental performance. Variations in injection volume can result in artificially high or low signal intensities compared to samples with correct amounts, potentially misrepresenting biological variability. For instance, if a test sample is injected with an incorrect volume while the PQC is injected with the correct volume, there is no way to detect this discrepancy solely through PQC analysis. Given that metabolomics often involves comparing metabolites between different groups (see section 1.1.2), these discrepancies can lead to erroneous conclusions.

Another significant drawback is the potential failure to detect less abundant metabolites in PQC samples due to their dilution. PQC samples are formed by pooling portions from each test sample (see section 1.3.1), leading to decreased concentrations of individual metabolites with increasing sample size. Consequently, certain metabolites that are present in some study samples may fall below detectable levels in PQC samples, impacting their inclusion in subsequent analysis. While these metabolites may still be detectable in individual study samples, their absence in PQC samples prevents their correction in data analysis.

Lastly, the use of pooled QC samples necessitates additional analysis runs, rendering it a time-intensive method. Because PQCs are prepared separately from test samples, they must be analyzed independently, adding to the overall analysis duration. Furthermore, PQCs must be included to correct for signal drift in instrumental analysis, which contributes to the complexity and time demands of this quality assurance approach.

1.4.2. Commenting on Matrix Effects in Global Metabolomics

Matrix effects are also a consideration in all analyses involving biological matrices (12). These effects occur within the ion source of a mass spectrometer, where co-eluting matrix

compounds can impact the ionization efficiency of analytes. This influence can either enhance the efficiency by increasing access to the surface of the droplet for gas-phase emission during the electrospray process (ion enhancement), or diminish it by competing with the analytes for the available charges (ion suppression) (28). In clinical chemistry, internal standards are commonly used to correct for matrix effect (12). However, this approach is impractical for global metabolomics due to the extensive number of metabolites involved (each requiring its own internal standard). Matrix effects are therefore generally accepted if they remain within acceptable limits that do not significantly affect the analyte.

1.5. Exploring the Potential of Isotope-labeled Internal Standards as Quality Assurance in LC-MS Metabolomics

Isotope-labeled internal standard (ILIS) is multipurpose in global metabolomics as they can be used to measure reproducibility of analytes, monitor signal responses throughout data acquisition, and detect potential outliers resulting from injection error or sample preparation issues (29). These standards involve substituting atoms within a molecule with stable isotopes such as ^{2}H (or D), ^{13}C, ^{15}N, or $^{17}O/^{18}O$ (14, 16), thereby increasing the mass of the standard (28). Despite sharing the same chemical formula and structure as their unlabeled counterparts, these isotopologs behave similarly during chromatography and are distinguishable by their greater mass in MS analysis, resulting in LC-MS peaks of higher mass (30).

Apart from their role in data quality assurance, ILISs also serve as effective indicators of instrument performance. An increasing trend in sensitivity might signal an issue with the autosampler, whereas a decreasing trend could for example indicate a possible leak (29).

1.5.1. The Use of ILISs for Data Quality Assurance

The use of ILIS holds the potential to provide rapid data quality assurance without relying on pooled QC samples (21). By incorporating ILIS into biological samples, key information on these labeled compounds, such as peak area and retention time, becomes readily available in every sample.

Repeatable peak areas of ILISs across different samples, accounting for expected minor variations due to matrix effects (see section 1.4.2), suggest that variations observed in

endogenous metabolites likely stem from biological differences (31). This is because the same quantity of ILISs should be present in each sample, having been added to the samples in equal amounts, whereas the levels of endogenous metabolites naturally differ. Figure 12 demonstrates how this can look graphically.

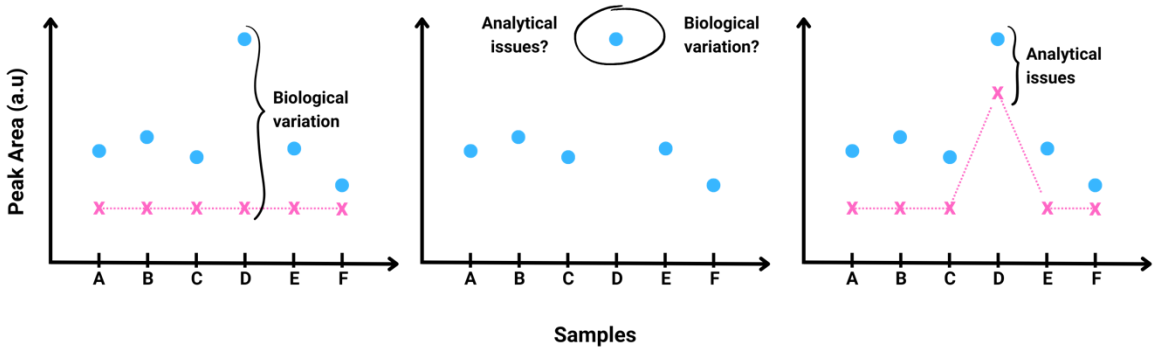


Figure 12 *Graphical representations of the role of isotope-labeled internal standards (ILIS) as a quality assurance tool in biological analysis. Pink "X" markers (X) represents ILIS signals, while blue circles (•) represent endogenous signals. The absence of ILIS signals in the middle graph makes it difficult to determine if variations of endogenous signals are due to biological variations or analytical issues. In the left graph, repeatable ILIS signals indicate that endogenous signals are biological. In the right graph, deviations in ILIS signals from repeatability suggest potential analytical issues among endogenous signals. Figure is made using Canva software.*

If the ILIS signals deviates from this repeatability it suggests that analytical issues are present. For example, if an ILIS signal significantly changes in conjunction with an endogenous signal, it often suggests specific instrumental issues like injection volume errors in that sample. These detailed assessments, not achievable with pooled QC samples, are crucial for promptly identifying potential errors and excluding problematic samples from broader analyses.

Relative standard deviation (RDS) and coefficient of variation (CV) are often used in analytical analysis as a measure of repeatability (24). They are both calculated using the standard deviation (SD) and the mean (μ), the difference being that CV is RDS multiplied by 100%. A low CV (typically below 10%) indicates consistent performance and minimal variation among the measured values. Equation 1 shows how CV is calculated.

$$CV = RSD\ in\ percent = 100\% \cdot \frac{SD}{\mu} \qquad (1)$$

To ensure the effectiveness of ILIS implementation, a robust analytical method and standardized sample preparation protocols is essential. These measures give confidence in the reliability of ILIS signals when interpreting results. In cases where questionable ILIS signal is observed the sample can be re-injected. Consistent detection of the same trend upon re-injection suggests the possibility of sample preparation errors, ruling out instrumental issues.

1.5.2. Strategies and Considerations in Designing ILIS Solutions for Global Metabolomics

Building upon the understanding of the role of ILISs in data quality assurance, effective strategies and considerations in designing ILIS solutions for global metabolomics are essential. Making an ILIS solution containing all possible metabolites that may be present in a sample used for global metabolomics is not feasible (32). Therefore, a compromise solution is needed, with the most common approach being to use a set of ILISs that represents the physiochemical properties of the entire analytical window. This includes considerations for both retention time and mass range, typically involving one ILIS per metabolite class. While some uncertainty arises for non-identical components, employing multiple ILISs with similar characteristics significantly enhances the reliability of quality assurance (33). Additionally, other factors such as availability and cost should be considered (21).

1.5.3. Applications of ILISs to Metabolomic Workflow

ILISs can serve various purposes within the metabolomic workflow, primarily focusing on system suitability assurance before, during, and after analysis, quality control during analysis, and post-analysis data correction (34). ILISs can be implemented either independently (typically unlabeled internal standards) or integrated into biological samples (often as isotope-labeled internal standards), with the latter approach preferred for quality assurance purposes.

For optimal performance in quality assurance and error correction, ILISs should be incorporated as early as possible in the process (31). Ideally, the ILIS solution should be introduced immediately after sample aliquoting. This ensures that all subsequent sample processing steps also involve the ILIS, allowing for the comprehensive tracking of any variability that may occur. Figure 13 illustrates the updated global metabolomic workflow with the integration of ILISs for quality assurance.

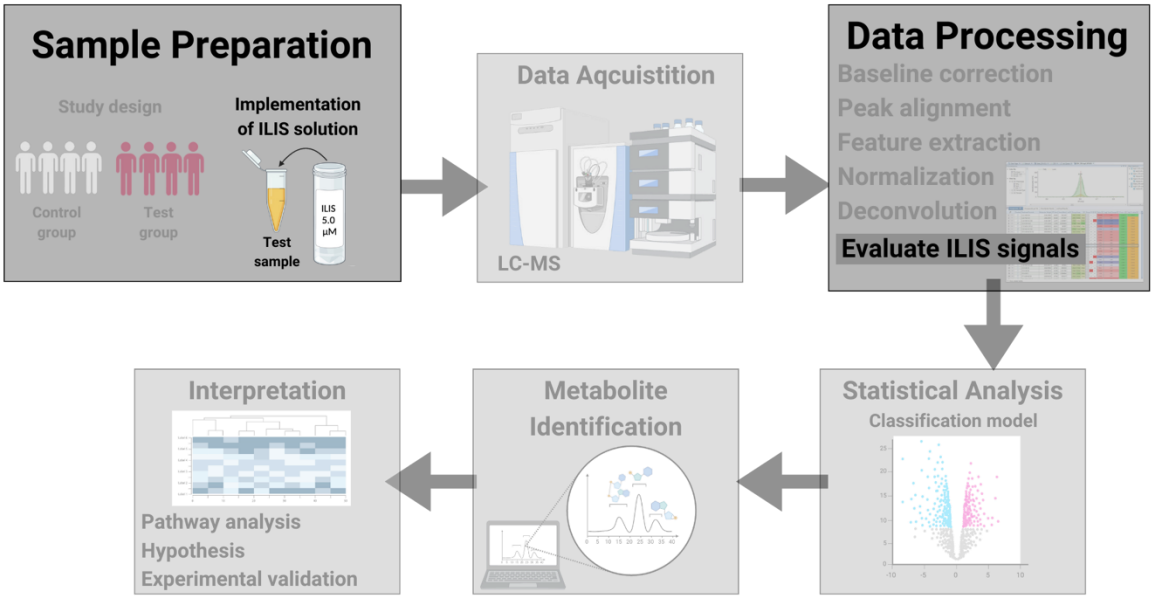


Figure 13 *The updated global metabolomic workflow after implementation of isotope-labeled internal standard (ILIS). The initial step of sample preparation now includes the addition of ILIS solution to the test samples. Moreover, the data processing step now involves the evaluation of ILIS signals before further analysis is done. Figure is adapted from (18, 19) using BioRender and Canva software.*

The ILIS signals are evaluated in the data processing step in the workflow. As illustrated in Figure 14, the initial stage involves verifying the repeatability of ILIS signals, typically indicated by low CV values, as mentioned in section 1.5.1. Any notable ILIS outliers should be identified during this phase. If such outliers are detected, a repeat analysis should be conducted to address potential analytical issues, such as spiking errors or instrumental anomalies. Upon confirming the repeatability of ILIS signals in the repeat analysis, the results can be reported. However, if repeatability is not observed, the samples should be re-prepared and re-analyzed.

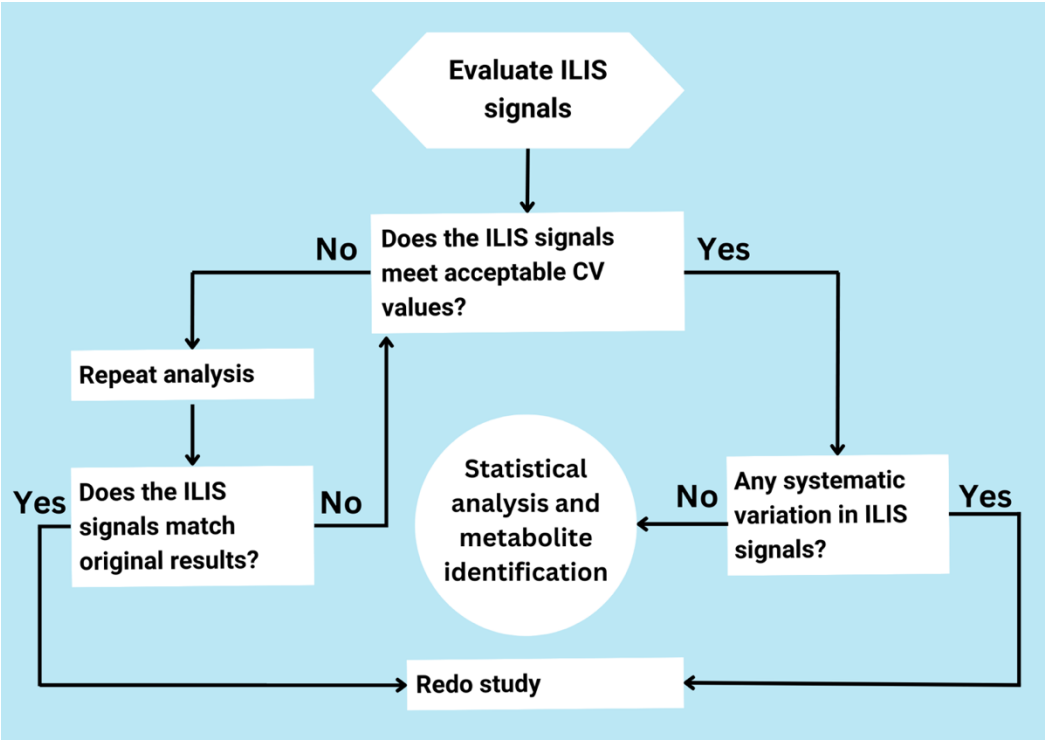


Figure 14 *Processing step in global metabolomic workflow after implementation of isotope-labeled internal standard as a quality assurance tool. If the ILIS signals meet acceptable coefficient of variation (CV) values, and there is no systematic variation in the signals, the workflows continue to statistical analysis and metabolite identification. Figure adapted from (31) using Canva software.*

1.6. Aim of Study

The primary aim of this study was to investigate whether the implementation of ILISs could enhance the quality assurance of global metabolomics analysis results. Specifically, the study aimed to facilitate easier and more precise comparison of metabolomic analysis results across various biological samples by ILISs.

To achieve this, the following research questions was addressed:

- Which ILISs should be chosen for the specific metabolomic analysis?
- How will the addition of ILISs impact the biological matrix?
- What is the comparative advantage of using ILISs over existing quality assurance methods in global metabolomics?
- How can the incorporation of these standards streamline the process of sample comparison and data interpretation in metabolomics studies?

The study involved preparation of a solution of carefully selected ILISs tailored to the metabolomic analysis. The ILIS solution was then added to biological samples (EDTA plasma, heparin plasma, serum, and DBS cards (see section 1.1.3)) followed by comprehensive LC-ESI-MS analysis to accurately profile metabolites. Finally, the data was evaluated to determine the effectiveness and practicality of using ILISs for quality assurance.

This research was significant as it addressed a critical need in global metabolomics by providing insights into the effective use of ILISs for enhancing data quality and reliability in global metabolomics. The anticipated outcomes included the development of guidelines for implementing ILISs into routine metabolomic workflow to ensure robust quality assurance, thereby advancing metabolomics research and applications.

2. Experimental

2.1. Small Equipment

Equipment used in the laboratory include: Fisherbrand™ Elite™ adjustable-volume pipettes and Fresco™ Microcentrifuge from Fisher Scientific (Waltham, MA, USA), Thermomixer Comfort from Eppendorf (Hamburg, Germany), Snap-top vials, Snap Ring Caps, and 0.1 mL Micro-Insert from Matriks AS (Oslo, Norway), AG 245 analytical balance weight from Mettler-Toledo (Columbus, OH, USA), and manual DBS puncher (3.2 mm) (from McGill (Jacksonville, FL, USA)).

2.2. Chemicals

2.2.1. Solvents and Reagents

Solvents and reagents used are presented in Table 1.

Table 1 Solvents and reagents used for the experimental part.

Name	Abbreviation	Purity (%)	Manufacturer
Solvents			
Methanol	MeOH	≥ 99.9	Rathburn Chemicals Lrd (Walkerburn, Scotland)
Formic Acid	FA	99	Thermo Fisher Scientific (Waltham, MA, USA)
Water*	H_2O	-	Merck (Darmstadt, Germany)
Reagents			
Alanine d4	Ala-d4	-	Cambridge Isotope Laboratories (Andover, MA, USA)
Tryptophan d5	Trp-d5	-	Sigma Aldrich (St. Louis, MO, USA)
C18 Acylcarnitine d3	C18-AC-d3	-	Larodan (Monroe, MI, USA)
Uracil $^{15}N_2$	U-$^{15}N_2$	-	Cambridge Isotope Laboratories (Andover, MA, USA)
EquiSPLASH™	-	-	Avanti® Polar lipids Inc (Alabaster, AL, USA)

***Note:** The water was type 1 water with a resistivity of 18.2 MΩ•cm at 25 °C provided from a Milli-Q-Purification system using a quantum cartridge and a 0.2 μm pore filter membrane from Merck (Darmstadt, Germany)

2.3. Solutions

Solutions used are presented in Table 2.

Table 2 Solutions and their compositions used for the experimental part.

Solution	Composition	Storage conditions
Extraction solution	80% MeOH, 20% H_2O, 0.1% FA	4 °C
Mobile Phase A*[1]	H_2O with 0.1% FA	Room temperature after preparation
Mobile Phase B*[1]	MeOH with 0.1% FA	
Calibration solutions*[2]	Pierce LTQ Velos ESI Positive/Negative Ion Calibration	-18 °C
Internal standard solution	See Table 3	-18 °C

***Note$_1$:** The mobile phases were prepared prior to each analysis.
***Note$_2$:** The calibration solutions are from Thermo Fisher Scientific (Waltham, MA, USA).

2.3.1. Preparation and Concentrations of Isotope-labeled Internal Standard Solution

Four isotope-labeled compounds (Ala-d4, Trp-d5, C18-AC-d3 and U-$^{15}N_2$) were individually dissolved and diluted with MeOH to achieve a concentration of 40 μM. Subsequently,

aliquots of each compound (40 µM) were mixed together to make a solution with 10 µM concentration containing all four compounds. This solution was combined with EquiSPLASH in a 1:1 ratio creating the stock ILIS solution, which was diluted with MeOH to four different concentrations (1:10, 1:100, 1:1000). All solutions were stored at -20 °C. The concentrations of the different ILIS-compounds are listed in Table 3.

Table 3 The concentrations of isotope-labeled internal standard (ILIS) components in stock ILIS solution.

Components	Formula	Concentration in stock-solution (µM)
Alanine (d4)	$C_3H_3D_4NO_2$	5.00
Tryptophane (d5)	$C_{11}H_7D_5N_2O_2$	5.00
Acylcarnitine C18 (d3)	$C_{25}H_{46}D_3NO_4$	5.00
Uracil $^{15}N_2$	$C_4H_4{}^{15}N_2O_2$	5.00
15:0-18:1 (d7) PC	$C_{41}H_{73}D_7NO_8P$	6.65
18:1 (d7) Lyso PC	$C_{26}H_{45}D_7NO_7P$	9.45
15:0-18:1 (d7) PE	$C_{38}H_{67}D_7NO_8P$	7.05
18:1 (d7) Lyso PE	$C_{23}H_{39}D_7NO_7P$	10.25
15:0-18:1 (d7) PG	$C_{39}H_{67}D_7NaO_{10}P$	6.55
15:0-18:1 (d7) PI	$C_{42}H_{75}D_7NO_{13}P$	5.90
15:0-18:1 (d7) PS	$C_{39}H_{66}D_7NNaO_{10}P$	6.45
15:0-18:1 (d7) TG	$C_{51}H_{89}D_7O_6$	6.15
15:0-18:1 (d7) DG	$C_{36}H_{61}D_7O_5$	8.50
18:1 (d7) MG	$C_{21}H_{33}D_7O_4$	13.75
18:1 (d7) Chol Ester	$C_{45}H_{71}D_7O_2$	7.60
18:1 (d9) SM	$C_{41}H_{72}D_9N_2O_6P$	6.75
C15 Ceramide (d7)	$C_{33}H_{58}D_7NO_3$	9.40

2.4. Biological Material

All samples analyzed in this study were taken from the Oslo University Hospital diagnostic biobank, or from healthy volunteers (REK approval 173346).

2.5. Sample Preparation

2.5.1. Sample Preparation of EDTA plasma for Concentration Optimization

Blood from a healthy individual was collected into three EDTA tubes and centrifuged at 3600 rpm for 10 minutes at 20°C. The resulting EDTA plasma was pooled, vortexed, and aliquoted into 1.5 mL Eppendorf tubes. Samples were stored at -80°C.

For EDTA plasma-ILIS samples, 30 µL EDTA plasma and 90 µL ILIS solution at various concentrations were combined in separate tubes, vortexed, and centrifuged at 14 800 rpm for 10 minutes at 4°C. EDTA plasma-MeOH samples followed a similar procedure, with 90 µL MeOH replacing the ILIS solution. Finally, the supernatant was aliquoted for analysis. The sample preparation for concentration optimization is illustrated in Figure 15.

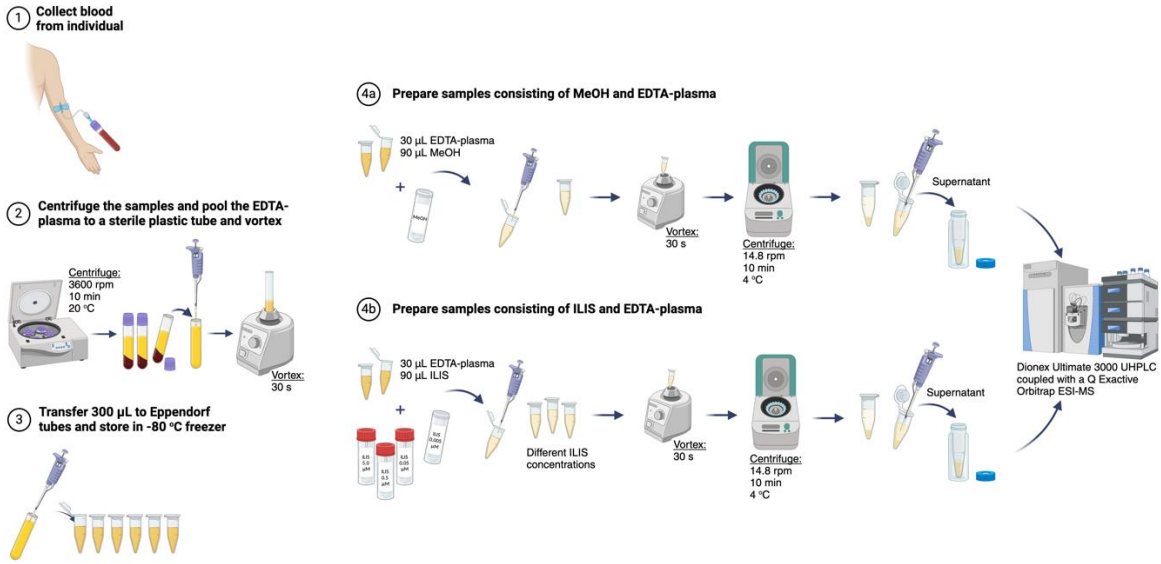


Figure 15 *Sample preparation of EDTA plasma for concentration optimization of isotope-labeled internal standard (ILIS) solution. EDTA plasma is collected and pooled before it is prepared for analysis. 4a) The sample preparation without addition of ILIS for comparison. 4b) The sample preparation with addition of ILIS before analysis. Figure is made using BioRender software.*

2.5.2. Sample Preparation of Patient EDTA plasma, Heparin plasma and Serum

Five patient samples each of EDTA plasma, heparin serum, and serum were obtained from the hospital laboratory. To each sample (30 µL), 90 µL of ILIS solution (5.0 µM or 0.5 µM) was added in separate Eppendorf tubes. After vortexing, the tubes were centrifuged at 14 800

rpm for 10 minutes at 4 °C. The supernatant was then transferred into vials for analysis. The sample preparation is illustrated in Figure 16.

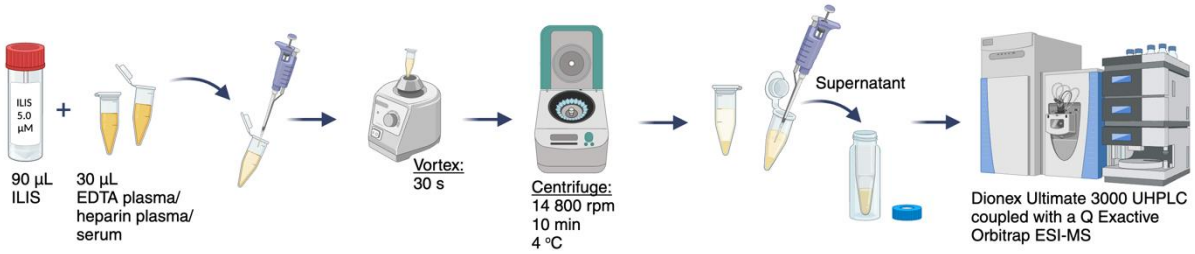


Figure 16 *Sample preparation of patient samples of clinically relevant matrices (EDTA plasma, heparin plasma, serum). After addition of isotope-labeled internal standard (ILIS) solution, the samples are vortexed and centrifuged. The supernatant is analyzed. Figure made using BioRender software.*

2.5.3. Sample preparation of Dried Blood Spots

Chosen DBS cards (Whatman 903™ Protein Saver Card) were initially spotted with 30 µL 5.0 µM ILIS solution. Each healthy volunteer was provided with two cards – one pre-spotted with ILIS solution and one non-spotted. Capillary blood was dripped onto both filter cards. On the pre-spotted cards, blood was dripped on top of the ILIS solution and left to dry for at least 4 hours. On the non-spotted cards, blood was dripped on the card, and two of five circles were spotted with ILIS solution on top of the blood, and left to dry (post-spotted cards with two blood spots with no ILIS solution). One punch from the pre-spotted cards and two punches from the post-spotted cards (one with ILIS solution and one with only whole blood) were transferred to separate Eppendorf tubes. To each tube, 100 µL of extraction solution was added (see Table 2), and mixed on a thermomixer at 700 rpm for 45 minutes at 4°C. The solution was then transferred to a sample vial for analysis. The sample preparation is illustrated in Figure 17.

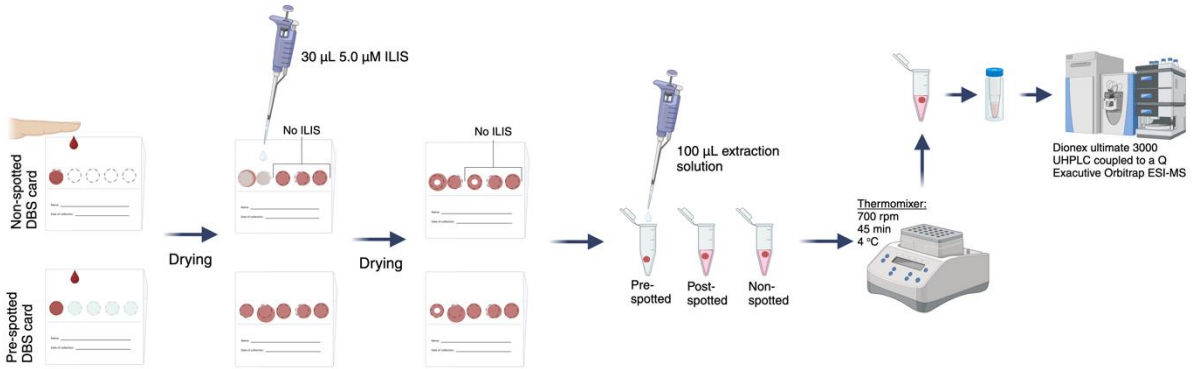


Figure 17 *Sample preparation of dried blood spot (DBS) cards. Two types were prepared – one pre-spotted with isotope-labeled internal standard (ILIS), and one non-spotted that was then post-spotted. A center punch was taken from the DBS cards, and extraction solution added. The samples were thermomixed and the supernatant analyzed. Figure made using BioRender software.*

2.6. Liquid Chromatography Mass Spectrometry – Instrumentation and Settings

A Dionex Ultimate 3000 UHPLC system, coupled with a Q Exactive Orbitrap ESI-MS (samples analyzed in both positive and negative ionization) from Thermo Fisher Scientific (Waltham, MA, USA), served as the standard instrumentation for all analyses. The analytical column utilized was the Pursuit XRs diphenyl (250 x 2.0 mm, particle size 3 μm) from Agilent Technologies (Santa Clara, CA, USA). The UHPLC and ESI-MS system settings, previously optimized in prior master's theses, were consistently utilized throughout this study for optimal compound separation and detection (35) (see Appendix 1).

2.7. Computer Software

Xcalibur (version 4.3) was used in the controlling of the LC-MS parameters, managing data acquisition, and generating sample sequences.

Tune (version 3.3) software was employed for calibrating the mass spectrometer in both positive and negative ionization modes. Calibration approval criteria included ensuring a variation in total ion count (TIC) of less than 10% relative standard deviation, achieving a TIC measurement exceeding 10^6 ions/second, attaining a base peak intensity surpassing 10^5 for the calibration solution, and maintaining mass accuracy below 3 ppm for external calibration and under 1 ppm for internal calibration. Additionally, Tune serves as the primary software for MS instrument control.

Freestyle (version 1.8) software was utilized for extracting ion chromatograms to observe peak areas and fragmentation spectra. To ensure successful ion extraction, a minimum mass accuracy above ± 5 ppm and peak areas exceeding 10^4 a.u. were required.

Excel (version 16.83) was employed for calculating standard deviations and the coefficient of variation (CV) using peak area data obtained from Freestyle. GraphPad Prism (version 10.2.2 (341)) was used for creating graphical representations of the data obtained from analysis.

3. Results and Discussion

3.1. Testing and Optimizing ILISs for Use in Global Metabolomics

3.1.1. Isotope-labeled Internal Standards Used

In the initial phase of this study, the use if ILISs in global metabolomics were tested and optimized. This process focused on selecting various isotope-labeled internal standards based on their retention- and mass area during analysis. Figure 18***Figure 18*** displays the ILISs chosen for the scan range of the method used (50-750 *m/z*). ILISs in the grey table are not detected and therefore not shown in the chromatogram given that their mass to charge ratio is above 750. Lipid analysis will not be extensively addressed in this study due to the method employed, however, their significance for subsequent lipidomic analysis should be acknowledged.

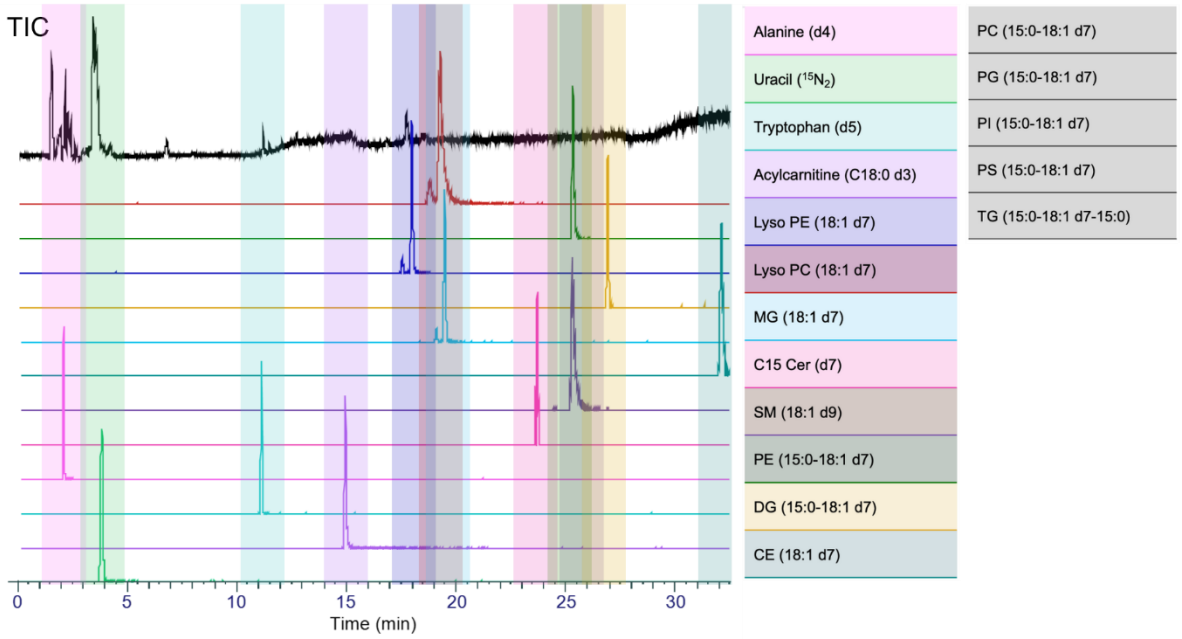


Figure 18 *The coverage of isotope-labeled internal standards (ILISs) within the scan range of the method (50-750 m/z), shown in extracted chromatograms (EIC) in positive ionization mode. The total ion count (TIC) is from EDTA plasma prepared with MeOH, while the EIC of alanine, etc. are ILISs from the 5.0 µM ILIS solution. Each ILISs are meant to cover a certain area of metabolites with similar characteristics. Figure made with Freestyle and Canva software.*

Note: *The colored columns are a predicted visualization and does not show a proven coverage of retention area.*

The total ion count (TIC) profile visible in black shows the elution pattern of endogenous metabolites present in EDTA plasma. To ensure improved quality assurance of all metabolites, it is imperative that the ILISs correspondingly covers these areas as comprehensively as possible (32). This consideration guided the selection of these specific ILISs.

In the initial phase of the analysis (1-5 min) there are a substantial number of metabolites eluting. The two ILISs Ala-d4 (RT = 2.1 minutes) and U-$^{15}N_2$ (RT = 3.9 minutes) elutes in this area, suggesting efficient coverage of these early eluting metabolites. Furthermore Trp-d5 (RT = 11.1 minutes) and C18-AC-d3 (RT = 15.0 minutes) covers a different part of the analysis, aligning with an increasing trend in metabolite elution observed as the analysis gradient becomes more organic. These four ILISs (Ala-d4, Trp-d5, C18-AC-d3 and U-$^{15}N_2$) are chosen due to their well-documented performance and characteristics, which are supported by extensive experience gained through their inclusion in the method's SST (see section 1.3.1). The familiarity and knowledge make these ILISs the primary focus of the study moving forward. The remaining time of analysis is covered by lipid ILISs, which were also included for future testing using global LCMS lipidomics. Notably, the lipidomic methods typically have a higher *m/z*-range due to larger masses of lipids, which would be more suitable for the lipid ILISs that was not detected here (36).

An important limitation in ILIS selection is the challenge to cover all metabolites detected. In global metabolomic analysis, this is not feasible due to the extensive number of metabolites eluting (32).

3.1.2. ILIS Concentration Optimization using EDTA Plasma

After solidifying which analytical standards to use for subsequent testing, the next step involved optimizing their concentration in a representative biological matrix. EDTA plasma, a commonly used matrix in global metabolomics, was selected for this purpose. Samples from a healthy individual was spiked with all four ILIS concentrations (5.0, 0.5, 0.05 and 0.005 μM) and six injections was analyzed. By using samples from the same individual, matrix effects were accounted for.

Figure 19 presents graphical representations of the distinct characteristics of ILISs across the different concentrations, revealing observed variations in peak intensities and repeatability among the ILIS concentrations (data is presented in Table 1, Appendix 2).

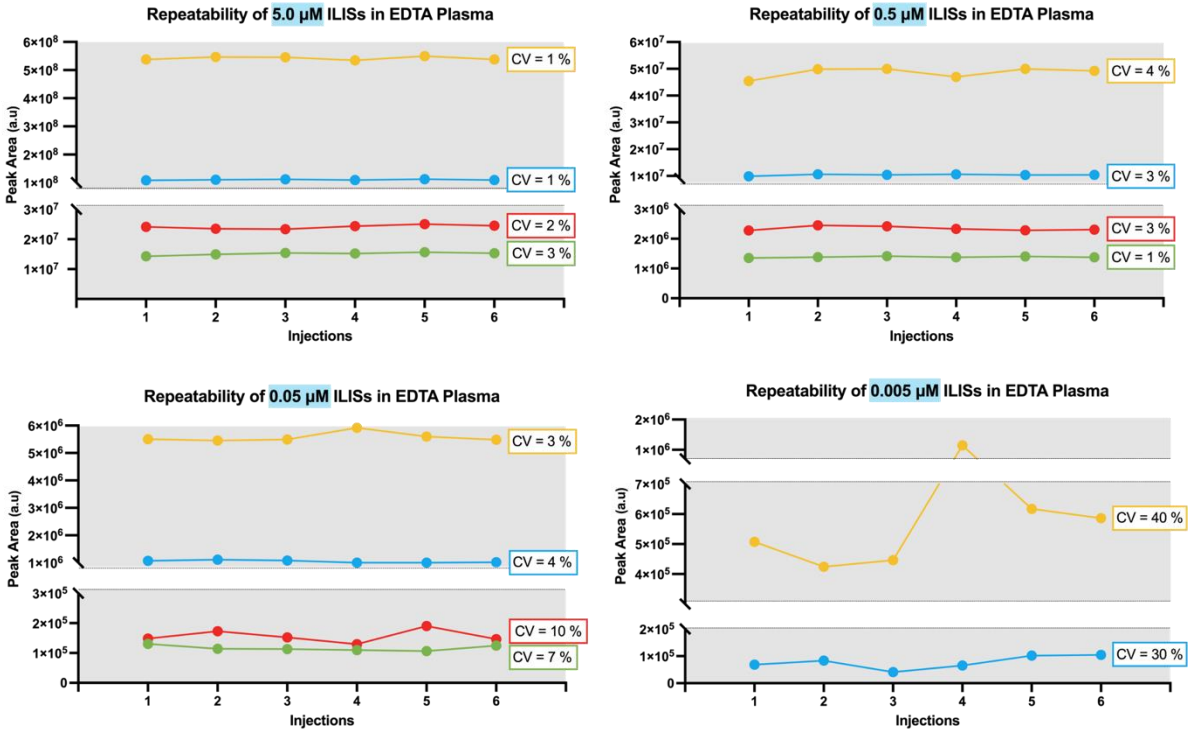


Figure 19 *Graphical representations illustrating the variation in peak intensities and repeatability of isotope-labeled internal standards (ILIS) solution across different concentrations. Peak areas are plotted against the number of injections, and the ILISs present are Ala-d4 (•), Trp-d5 (•), C18-AC-d3 (•), and U-$^{15}N_2$ (•). Ala-d4 and U-$^{15}N_2$ is not present in the 0.005 µM graph given there were no detectable signals. Plots were generated using GraphPad Prism software.*

Signal Strength Variability Between ILIS Concentrations

The peak intensities in the ILIS solutions vary in signal strength between different concentrations. At concentrations of 5.0 µM and 0.5 µM, robust signals are observed with peak areas ranging from above 1E10^6 to above 6E10^8 a.u. (analysis data is presented in Appendix 2). However, as the concentration decreases to 0.05 µM, the peak areas drops to 1E105-2E10^5 a.u. for Ala-d4 and U-$^{15}N_2$, and above 5E10^6 a.u. for C18-AC-d3. Notably, signals must fall within an optimal range – not too high to avoid saturation and not too low to ensure detectability – which is further discussed in subsequent paragraphs (37). At the lowest concentration of 0.005 µM, Ala-d4 and U-$^{15}N_2$ exhibit no detectable signal, while C18-AC-d3 and Trp-d5 display weak signals around 6E10^5 and below 1E10^5 a.u., respectively.

The results demonstrate that higher concentrations of ILIS solution yield greater peak areas, as expected. Specifically, the signal strength between 5.0 μM and 0.5 μM has a factor of 10, indicating a linear concentration area (24). However, this linear relationship does not hold for concentrations of 0.05 μM and 0.005 μM, where signal become unrepeatable and non-detectable. It is crucial that these ILIS signals exceed the limit of detection (LOD) defined by the results (set at $6E10^4$ a.u.) to ensure effective quality assurance (see section 1.2.1). In addition, signals falling below this limit can introduce noise and uncertainty, potentially affecting the accuracy of metabolite detection (12). As the signals are low or not existent for ILIS solution concentrations of 0.05 μM and 0.005 μM, these concentrations are considered not to be sufficient for the intended purpose.

Notably, excessively high signal intensities should also be avoided, as they can increase the likelihood of matrix effects that can compromise the signal strength of co-eluting components (12). In targeted analysis, ILIS signals ideally closely resemble the analyte they are meant to correct for, generally falling within an acceptable range of ±2 SD (37). However, in global metabolomic analysis where multiple metabolites elute simultaneously, various factors can influence the SD. This could potentially affect the reliability of the ±2 SD range. Despite this, the ±2 SD range remains as an acceptable measure in this study in absence of a better alternative, along with CV values.

Instrumental Repeatability at Different ILIS Concentrations

The optimization of ILIS concentration in EDTA plasma from the same individual ensured consistent matrix effects across injections (12). Consequently, any observed variation can be confidently attributed to factors other than matrix effects. Based on this, the ILISs in solutions of 5.0 μM and 0.5 μM have consistently low CV values ranging from 1% to 4%, calculated using Equation 1. At concentration of 0.05 μM, the CV values for Ala-d4 and U-$^{15}N_2$ are slightly elevated at 7% and 10%, while the lowest concentration of 0.005 μM has remarkable higher values of 30% and 40% for Trp-d5 and C18-AC-d3.

Most ILIS concentrations show CV values under or equal to 10% except the lowest concentration at 0.005 μM. These CV values reflect low variation between injections and indicates consistent and repeatable signals, which are essential for using ILISs as a quality assurance measure (31). Repeatable signals ensure that any observed changes in signals are attributable to instrumental errors rather than fluctuations in the ILIS solution itself. For

example, the CV values for the lowest concentration (30-40%) indicate significant variability between injections. Using such low ILIS concentrations for quality assurance would make it impossible to know if an observed variation is due to analytical issues or the ILIS' lack of repeatability.

Relation between ILIS/Endogenous Ratios and Concentration

One final aspect of investigating ILIS solution optimization involves examining the ratios between ILIS signals and endogenous signals. For this purpose, the same EDTA plasma samples as above with different concentrations was used. The ratios are presented in in Table 4, and shows a consistent trend where higher concentrations of ILIS solution correspond to lower ratios between ILIS and endogenous signals.

Table 4 Ratios between isotope-labeled internal standards signals and endogenous signals. Not detected (N.D.)

Ratio	5.0 µM	0.5 µM	0.05 µM	0,005 µM
Ala-d4 / Ala	3.0E+01	3.2E+02	3.9E+03	N.D.
Trp-d5 / Trp	5.0E+00	5.3E+01	5.2E+02	7.9E+03
C18-AC-d3 / C18-AC	1.9E-03	2.1E-02	2.1E-02	1.8E+00
U-15N2 / U	2.0E-01	2.1E+00	3.0E+01	N.D.

This trend suggests that higher ILIS concentrations lead to ILIS signals that closely resemble the endogenous signals, which is desirable for targeted analysis. However, this alignment may not be transferable to global analysis due to the simultaneous elution of numerous compounds, which could be affected by the ILIS' presence. Regardless, in absence of a better alternative, the same alignment is interpreted as there are considerable experience with targeted analysis. In the future, further testing of ILISs could be done to investigate possible impacts on other co-eluting metabolites.

3.1.3. Impacts of ILISs on Endogenous Metabolites

Following the determination of the optimal ILIS concentration, the potential impact of adding ILIS solution on the metabolome was evaluated with the aim of minimizing disturbance to endogenous metabolite signals. The results of differences in peak areas are illustrated in Figure 20, specifically for Ala-d4.

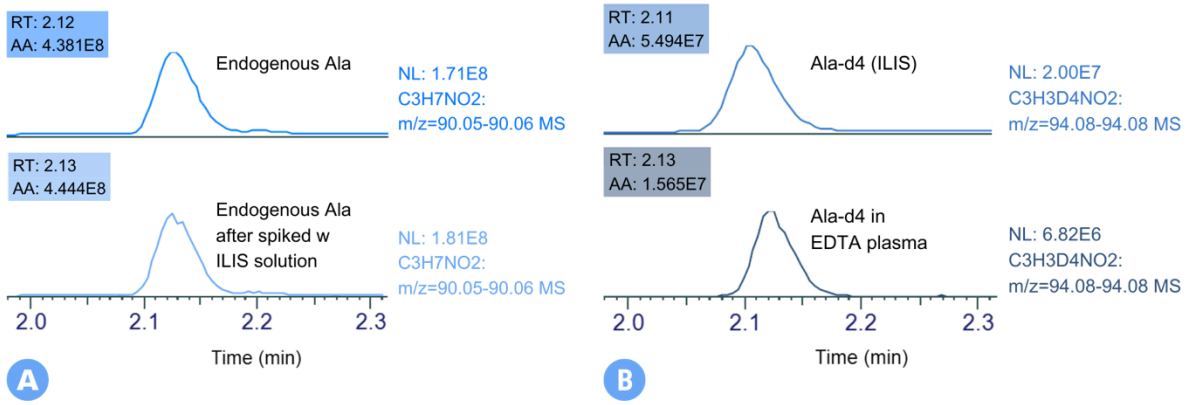


Figure 20 *A) The negligible impact of the isotope-labeled internal standards (ILIS) solution on endogenous alanine with a ratio of 1.0 (Ala/Ala-d4). B) The impact on Alanine-d4 when added to EDTA plasma with a ratio of 0.3 (Ala-d4 in matrix/Ala-d4). The figure is made using Freestyle and Canva software.*

The peak area of endogenous alanine is 4.381E8. After adding the 5.0 µM ILIS solution, the peak area slightly increases to 4.444E8, resulting in a ratio of 1.0 (Ala/Ala-d4). This finding suggests that the ILIS solution has a negligible impact on the endogenous signal, indicating potential applicability to other compounds in this elution area where many polar components co-elute simultaneously with alanine (32). Furthermore, the peak area of the ILIS Ala-d4 (5.0 µM) is 5.494E7. When adding the 5.0 µM ILIS solution to EDTA plasma, the peak area decreases to 1.565E7 as expected, resulting in a ratio of 0.3 (Ala-d4 in matrix/Ala-d4).

Summary part 3.1.: The strategically selection of isotope-labeled internal standards (ILISs) based on retention times aligns with the metabolite elution pattern in the LC-MS analysis. While ILIS coverage extends to many metabolites, limitations arise in regions without ILISs representation. Following the selection of ILISs, four concentrations of ILIS solution revealed different results regarding signal strength, repeatability and ratios between ILIS and endogenous metabolites. Concentrations of 5.0 µM and 0.5 µM demonstrated robust signals and low variation between injections, ensuring repeatable signals. A linear relationship was also observed between these concentrations, indicating a consistent response in signal strength. Furthermore, the addition of 5.0 µM ILIS solution was proven to have negligible impact on endogenous signals, with a ratio of 1.0 (Ala/Ala-d4). The ILIS signal itself decreases when added to EDTA plasma (ratio of 0.3 (Ala-d4 in matrix/Ala-d4)).

In conclusion, concentrations of 5.0 µM and 0.5 µM showed the most promising results and are therefore further used in this study.

3.2. The use of ILISs in Clinically Relevant Matrices

Once the ILIS solution was optimized, the application of it across various clinically relevant matrices, including EDTA plasma, heparin plasma, serum, and DBS cards, was explored. To do this, patient samples from the hospital labs were used. Notably, the order of injections of the samples were randomized, however the possibility of decreasing/increasing effects in instrumental performance was investigated and neglected.

3.2.2. Impact of ILISs – Insights from EDTA Plasma Patient Samples

When spiking five EDTA plasma patient samples with 5.0 µM ILIS solution, all peak area signals were above the detection limit. Figure 21 shows graphical representations of the signals for both ILISs and endogenous metabolites. Notably, the ILIS signals are withing the acceptable range of ±2 SD endogenous signals for all four ILISs, as described in section 3.1.2. This means that the ILISs have a good starting point to act as quality assurance (31). C18-AC-d3 signals are at the edge of this threshold with high signals, indicating that the concentration of this ILIS should be advantageously adjusted lower to get closer to the endogenous signals, as shown in section 3.1.2.

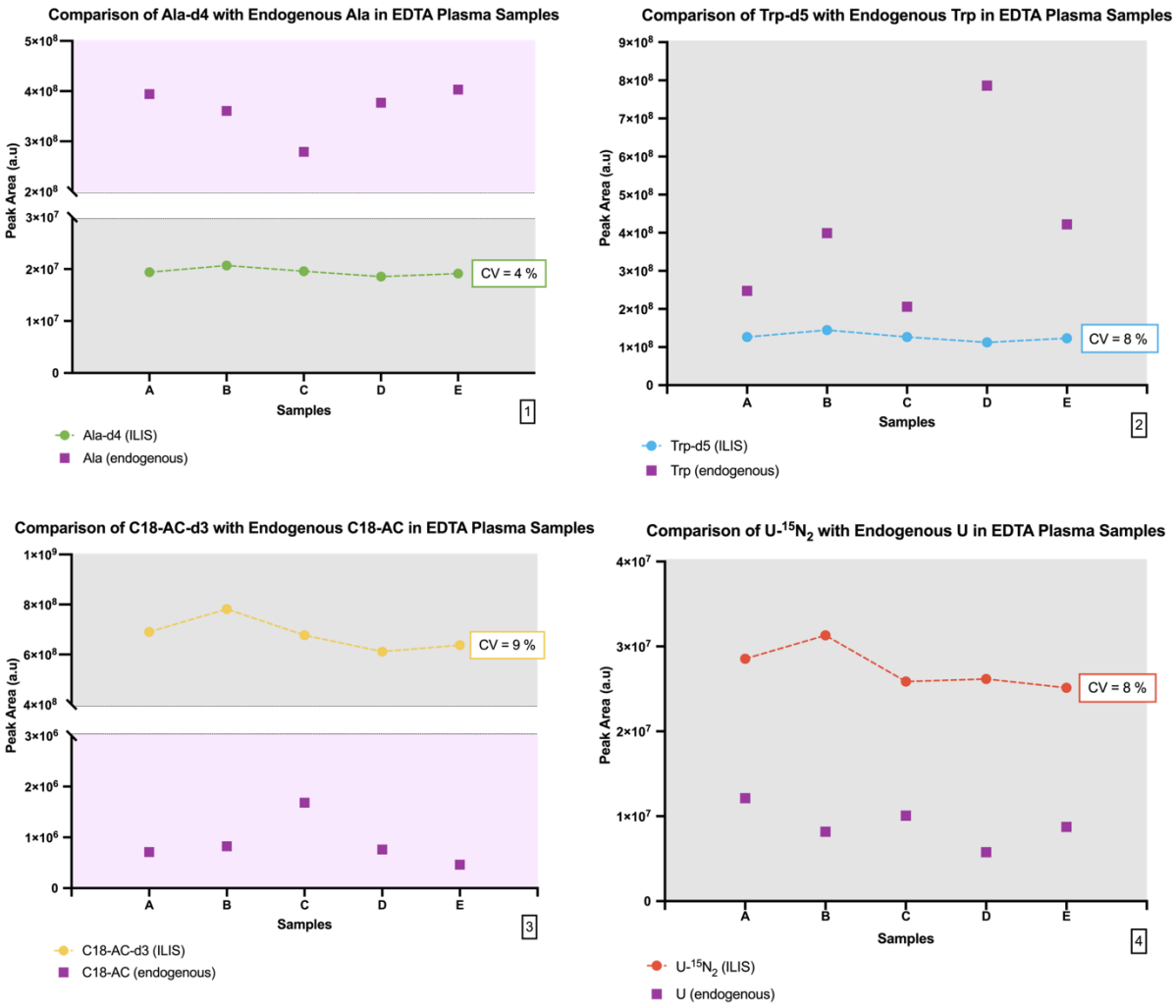


Figure 21 *Graphs depicting peak areas of isotope-labeled internal standards (ILISs) and endogenous metabolites in EDTA plasma samples A-E spiked with 5.0 µM ILIS solution. Graphs were generated using GraphPad Prism software.*

To ensure that the ILISs serve their purpose as quality assurance measures, it is crucial that their signals have minimal variations influenced by matrix effects (12, 31). To assess the repeatability of ILIS signals, CV values are calculated using Equation 1 and presented in Figure 21. Notably, all CV values are below 10% for the ILIS signals, indicating consistent and repeatable ILIS signals.

Another method to demonstrate the repeatability of ILIS signals involves comparing the signals of the different ILISs between different samples and looking at the trend. Since the concentration of each of the four ILISs are similar, their signals should be consistent in the different samples. This means that if sample B has higher Trp-d5 signals compared to A, it should also have higher Ala-d4 signals compared to A. Figure 22 shows that this is the case,

confirming the repeatability of ILIS signals. Moreover, the heightened signal of sample B can be explained by less ion suppression affecting this sample, or differences in pipetting (12).

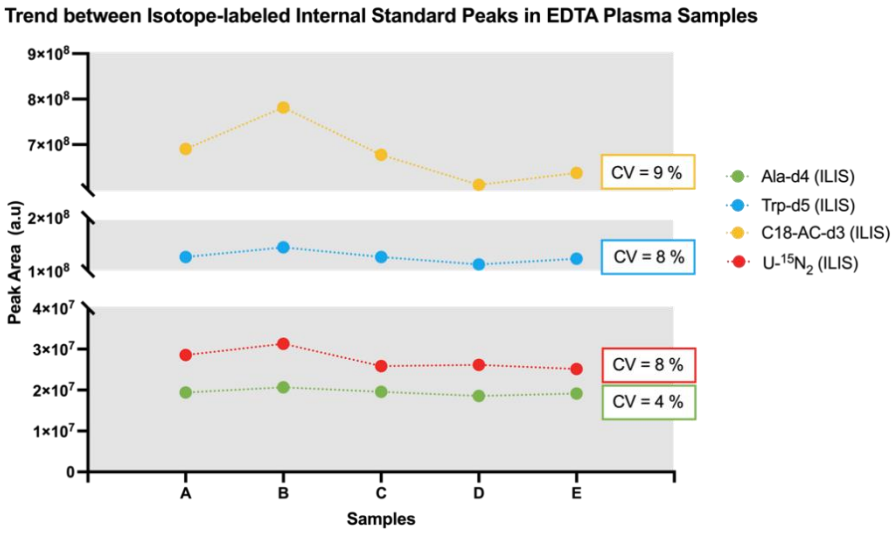


Figure 22 *Graph depicting trends in isotope-labeled internal standard (ILIS) responses across different EDTA plasma samples A-E, supported by consistent coefficient of variation (CV) values. The graph was generated using GraphPad Prism software.*

When using the ILIS signals as quality assurance, the ILIS signals are used to correct the endogenous signals (31). This approach ensures that any observed variations in endogenous signals are attributed to biological factors. For instance, the stable signal observed for Trp-d5 in Figure 21 graph 2 confirms that the elevated endogenous signal observed in sample D reflects biological variability rather than analytical issues. If an analytical issue were present, the ILIS signals would be deviant from the other ILIS signals of other samples, leading to insecurities.

To validate the authenticity of endogenous variations, their concentrations were assessed using targeted analysis conducted in the routine hospital laboratory. The results, presented in Table 1 in Appendix 3, confirm that the observed variations between samples are consistent and thereby reflect genuine biological differences.

3.2.3. Impact of ILISs – Insights from Heparin Plasma Patient Samples

The investigation into heparin plasma samples spiked with 5.0 µM ILIS solution mirrored the findings in EDTA plasma. Graphical representations are shown in Figure 23. Similar to EDTA plasma, all peak area signals in heparin plasma were above the (see section 1.2.1). All four ILISs fell within the acceptable range of ±2 SD of endogenous signals, as described in

section 3.1.2. Notably, the adjustment of C18-AC-d3 to lower concentrations could optimize its proximity to endogenous signals.

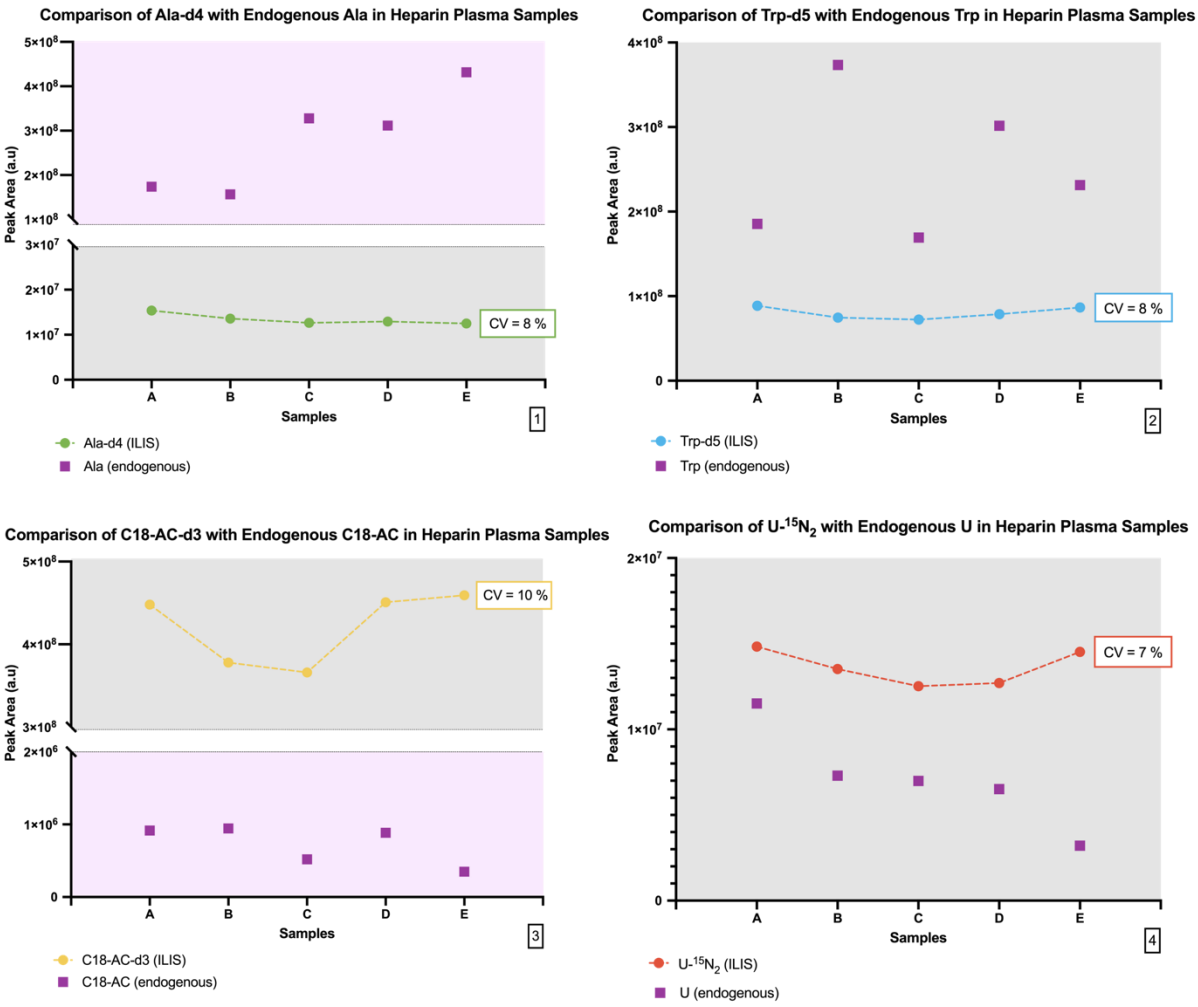


Figure 23 *Graphs depicting peak areas of isotope-labeled internal standards (ILISs) and endogenous metabolites in heparin plasma samples A-E spiked with 5.0 µM ILIS solution. Coefficient of variation (CV) values demonstrate sample variation. Graphs were generated using GraphPad Prism software.*

As for the repeatability of ILIS signals, the CV values were calculated using Equation 1 and are shown in Figure 23. All CV values are within acceptable values, indicating repeatable ILIS signals (31). Further confirmation of signal repeatability was obtained by comparing different ILIS signals between different samples (Figure 24). Lower signals in sample B and C suggests greater ion suppression effects in these samples (12).

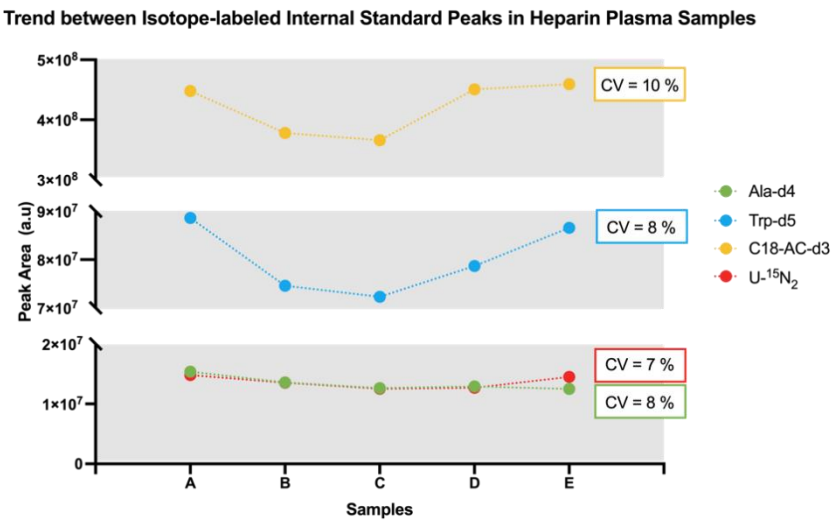


Figure 24 *Graph depicting trends in isotope-labeled internal standard (ILIS) responses across different heparin plasma samples A-E, supported by consistent coefficient of variation (CV) values. The graph was generated using GraphPad Prism software.*

The findings suggest that ILISs can effectively serve as a quality assurance tool in heparin plasma, ensuring that any observed variations in endogenous signals are attributed to biological factors. For instance, the repeatable signal observed for Ala-d4 in Figure 23, graph 1 supports the conclusion that variations in endogenous Ala levels are biological rather than from analytical issues.

Additionally, validation through targeted analysis of endogenous concentrations (Table 2, Appendix 3) confirms consistent variations between samples and reflects genuine biological differences.

3.2.4. Impact of ILISs – Insights from Serum Patient Samples

The next matrix tested was serum. This is the second component of blood, which is frequently utilized alongside plasma in metabolomic analyses (7). Upon analyzing serum patient samples spiked with 5.0 µM ILIS solution, all peak area signals were above the LOD (see section 1.2.1). In addition, three out of four ILISs fell within the acceptable range of ±2 SD of endogenous signals (see section 3.1.2). C18-AC-d3 did not fall within this range. Figure 25 shows the graphical representations of the results.

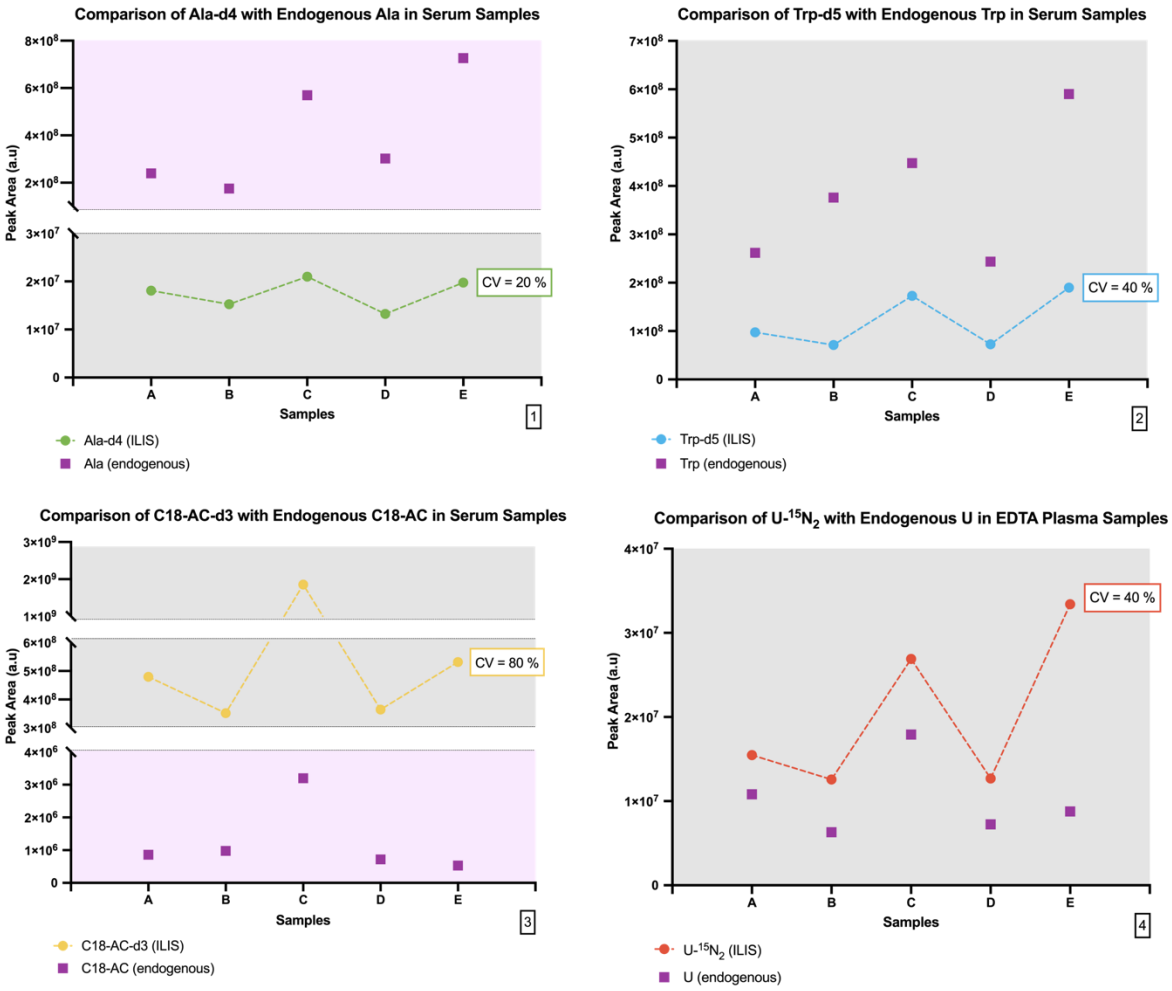


Figure 25 *Graphs depicting peak areas of isotope-labeled internal standards (ILISs) and endogenous metabolites in serum samples A-E spiked with 5.0 µM ILIS solution. Coefficient of variation (CV) values demonstrate sample variation. Graphs were generated using GraphPad Prism software.*

The results reveals that the ILIS signals are not repeatable, reflected by CV values of 20-80%. This raises concerns in regards to possible analytical issues, and further investigations must be done to evaluate these results.

To start out, samples C and E exhibited significantly higher signals for endogenous metabolites Ala (alanine) and Trp (tryptophan) compared to the other samples. Sample C, in particular, showed elevated signals across all endogenous metabolites. This discrepancy raises questions about whether these variations stem from biological factors, a matter which could be corrected using ILISs (31). The elevated ILIS signals observed in sample C compared to the other samples suggest an unusually high injection volume specific to sample C. Consequently, both the ILIS signal and endogenous signal appear higher than expected for this sample. This anomaly likely contributes to the outlier of sample C, with variations in its

signals likely stemming from non-biological factors. Because of this, sample C is further investigated for quality assurance.

Firstly, the peak area integration for sample C was validated. Subsequently, other injections of the same sample were compared to the sample with the outlier present. For this purpose, both incorporating negative ionization data and/or a lower ILIS concentration spike could be used. In a scientific study, the same sample would be reinjected for further validation. Figure 26 illustrates results from the injection of the same sample but spiked with a 0.5 µM ILIS solution.

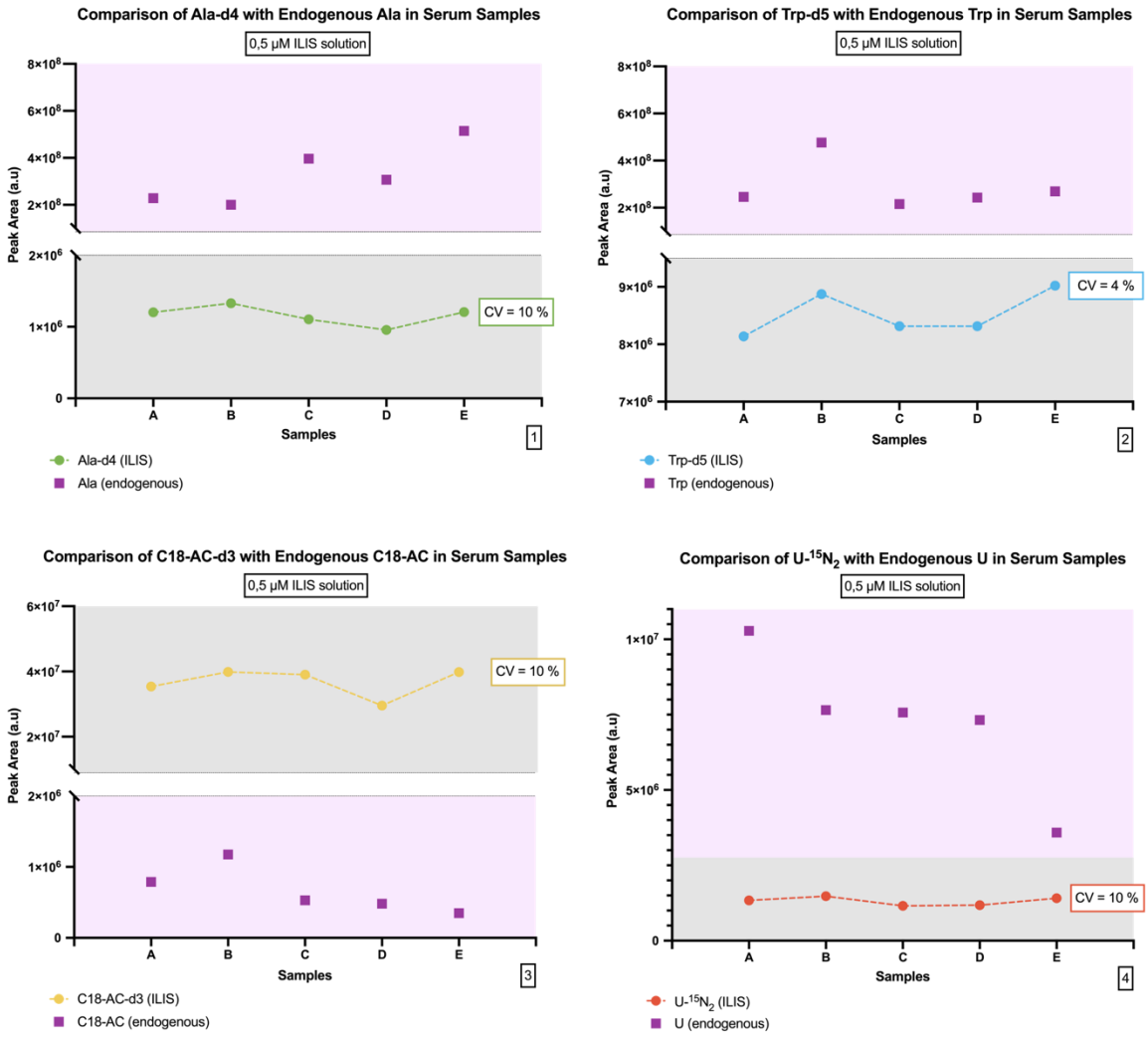


Figure 26 *Graphs depicting peak areas of isotope-labeled internal standards (ILISs) and endogenous metabolites in serum samples A-E spiked with 0.5 µM ILIS solution. Coefficient of variation (CV) values demonstrate sample variation. Graphs were generated using GraphPad Prism software.*

These graphs indicate that sample C no longer appears as an outlier, with lower peak areas compared to the previous injection and reduced variations relative to other samples. In

addition, ILIS levels now demonstrated minimal variation among samples with CV values of 4-10%, similar to the findings in EDTA and heparin plasma matrices. This serves as proof of concept, highlighting the importance and effectiveness of implementing ILISs in this study, as it functions as intended.

To substantiate these findings, the endogenous concentrations from targeted analysis in the routine hospital lab was compared (Table 5). The table is a combination of Table 2 and Table 3 from Appendix 3 as Ala and Trp was not measured in the serum samples D and E. Since it is the same patient, levels of Ala and Trp in heparin will reflect the levels in serum as well.

Table 5 Endogenous concentrations of patient samples obtained by targeted analysis from the Oslo University Hospital diagnostic biobank.

Sample ID	Concentration (µmol/L)			
	Ala	Trp	C18-AC	U
A_S	231	40	not measured*	not measured*
B_S	175	83	not measured*	not measured*
C_S	432	36	not measured*	not measured*
D_S	335	47	0.042	not measured*
E_S	508	33	0.029	not measured*

***Note:** Not all metabolites are measured for all the samples in the hospital routine lab as analyses are tailored based on the patient´s clinical needs and symptoms specified in the doctor's requisition.

From Table 5, it is evident that the concentration of sample C does not consistently exceed that of other samples for all four metabolites. While Ala concentration is higher than samples A, B, and D, it is not as pronounced as initially observed. In addition, the Trp concentration in sample C is notably lower than in samples A, B, and D, contrary to the initial 5.0 µM injection of this sample. This suggests that the high endogenous metabolite signals observed in sample C from the 5.0 µM ILIS spike, does not reflect actual endogenous concentrations accurately. Instead, they are most likely due to analytical issues. This proves that the use of ILISs can showcase analytical errors in single injections in contrast to PQCs where quality assurance for single samples is very difficult, as mentioned in section 1.4.1.

3.2.5. Impact of ILISs – Insights from DBS-cards Samples

The final matrix to be tested is whole blood using DBS cards. In EDTA plasma, heparin plasma and serum the ILISs was added to the extraction solution (see section 2.5.2). Now, the question of interest is whether it is possible to directly add ILIS to DBS cards by spotting them with ILIS solution either before or after blood spotting. When implementing this method, all peak area signals were above the LOD (see section 1.2.1), and all four ILISs were within the acceptable range of ±2 SD of endogenous signals (see section 3.1.2), for both cards spotted prior to and after blood spotting. Graphical representations are depicted in Figure 27.

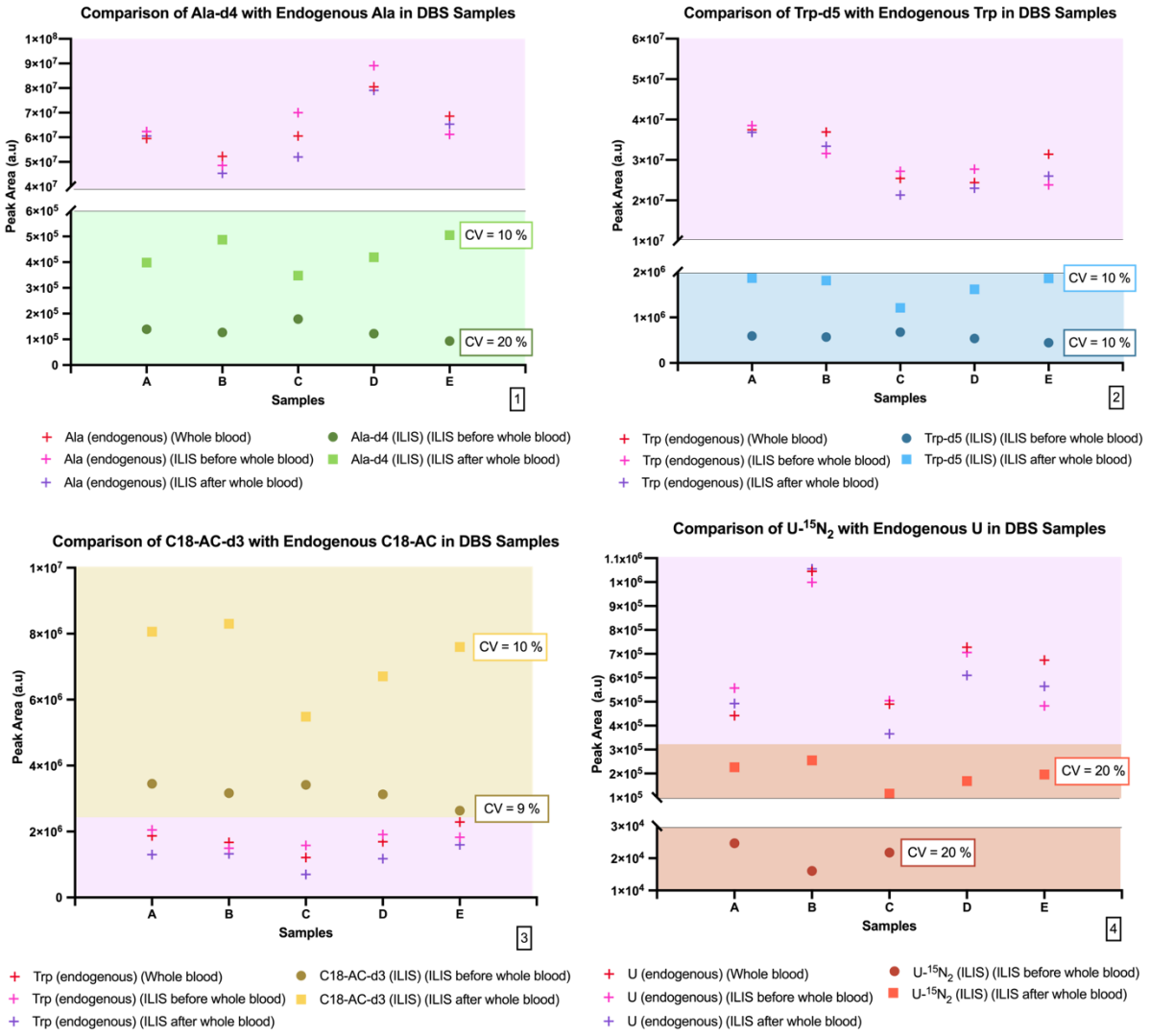


Figure 27 *Graphs depicting peak areas of isotope-labeled internal standards (ILISs) and endogenous metabolites on dried blood spot (DBS) cards spiked with ILIS solution both prior to and after blood addition. Endogenous metabolites are depicted for whole blood (+ red cross), DBS cards spiked with ILIS before blood addition (+ pink cross), and DBS cards spiked with ILIS after blood addition (+ purple cross). Coefficient of variation (CV) values demonstrate signal repeatability. Graphs were generated using GraphPad Prism software.*

The repeatability of ILISs is enhanced when cards are spotted after the addition of whole blood. For most ILISs, except U-$^{15}N_2$, the CV value remains consistently at 10%, signifying acceptable repeatability. Although U-$^{15}N_2$ shows a slightly higher CV value, it is still not a concerningly high CV given that the CV increases with decreasing signals.

Cards spotted with ILIS prior to blood spotting also exhibit relatively repeatable ILIS signals, with CV values ranging from 9% to 20%. Furthermore, the signal repeatability is confirmed by comparing ILIS signals within the same sample for both card types (Figure 28).

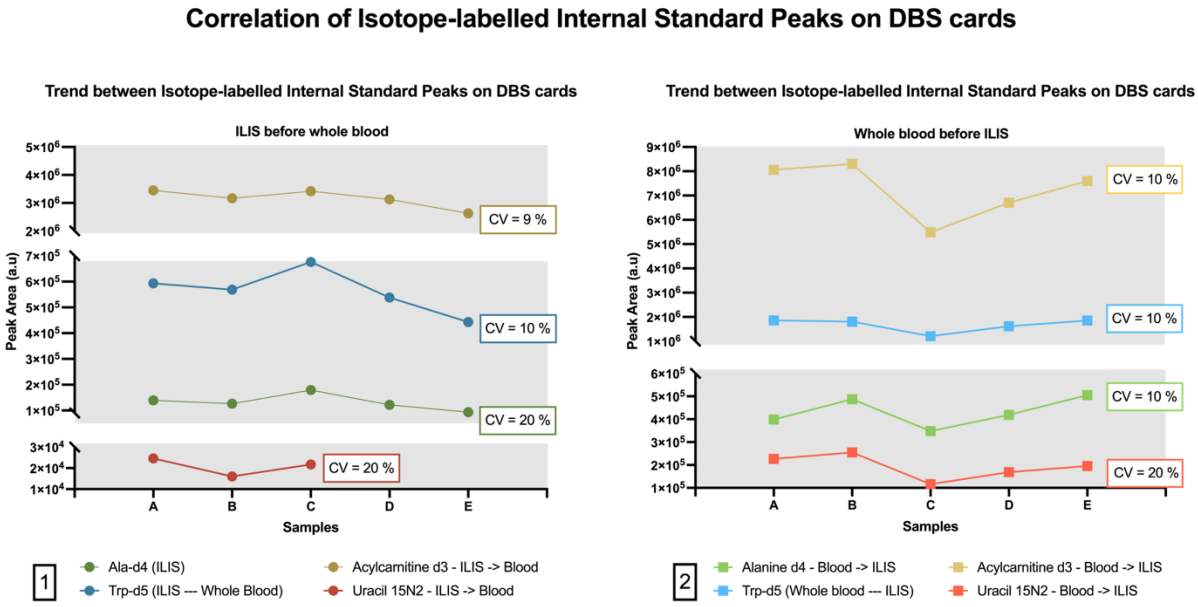


Figure 28 *Graphs depicting trends of isotope-labeled internal standard (ILIS) and endogenous metabolite signals across different dried blood spot (DBS) samples spiked with ILIS solution. For graph 1, ILIS solution is spotted before blood spotting, while for graph 2, ILIS solution is spotted after blood spotting. Graphs were generated using GraphPad Prism software.*

Both spotting methods demonstrate potential suitability for utilizing ILIS as a quality assurance tool. However, cards spotted after blood application display ILIS signals that closely resemble endogenous signals, contributing to enhanced stability. Additionally, the ILIS signals are higher for cards spotted after blood spotting. As a result, there is more variation in ILIS signals on cards spotted before blood, as reflected in the CV values of the lowest signals in Figure 28, graph 1. Based on these findings, it is preferable to spot the cards with ILIS solution after the addition of blood to achieve less variation due to the higher signals.

A potential limitation of this approach is that it solely assesses the direct spotting of ILIS onto

the DBS cards. Future research could explore the efficacy of adding ILIS solution to the extraction solution, potentially yielding improved results compared to direct spotting onto the cards.

Summary part 3.2.: Clinically relevant matrices was used to test the impact of 5.0 µM ILIS solution. When spiking EDTA- and heparin plasma with 5.0 µM ILIS solution repeatable ILIS levels was demonstrated (CV values: 4-10% among samples). In serum samples, high variation in ILIS signals was observed, as well as an endogenous outlier (sample C). This raised concerns about analytical issues, and further investigation of sample C was done. These results lead to no outlier in sample C, and acceptable CV values of ILIS signals, serving as a proof of concept and highlighting the importance of implementing ILISs in global metabolomics. It also proves that addition of ILIS in sample preparation can be used in global metabolomics.
Moreover, the decision on whether to spot DBS cards prior to or after blood spotting reveled that both spotting methods holds potential. Nevertheless, spotting with ILIS after blood addition yielded higher ILIS signals with lower variations and closer resemblance to endogenous signals. Therefore, spotting with ILIS on DBS cards should be conducted after blood addition to optimize signal quality and repeatability.

4. Conclusion

This study firmly establishes the crucial role of ILISs in improving quality assurance within global metabolomic analysis. Through optimization of a solution made by carefully selected ILISs, comprehensive coverage of the endogenous metabolites eluting in the employed global metabolomic method was achieved. Specifically, concentrations of 5.0 µM and 0.5 µM emerged as the optimal ILIS concentrations, consistently delivering robust and repeatable signals within EDTA plasma. Furthermore, the addition of the ILIS solution had a negligible impact on endogenous signals, with a ratio of 1.0 for endogenous Ala compared to Ala-d4.

Moreover, the investigation into clinically relevant matrices highlighted the essential nature of ILISs in distinguishing analytical issues from biological variance. When spiked into patient samples of EDTA- and heparin plasma, repeatable ILIS signals ensured that any observed variation in endogenous signals was attributed to biological factors rather than

analytical issues. In contrast, when spiked into serum samples, non-repeatable ILIS signals drew attention to an endogenous outlier of sample C. Analyzing other injections of this sample revealed that the outlier stemmed from analytical issues, and not biological variance. This serves as proof of concept and showcase the ILISs' capability to identify analytical errors for individual samples or injections – a capability which has not been possible before.

To further expand the utility of ILISs, their application on DBS cards was explored. By spotting with ILIS solution after spotting with whole blood, the ILIS signals were repeatable and came close to the endogenous signals. This investigation highlights the potential of ILIS application on DBS cards.

In conclusion, the study confirms that the ILIS solution used can serve as a quality assurance tool in global metabolomics, surpassing existing methods.

4.1. Future Work

In future studies, the concentrations of ILISs in the solution should be adjusted and optimized to make the ratios between ILIS and endogenous signals decrease. Additionally, exploring the use of different ILISs could further improve coverage of endogenous metabolite elution.

Furthermore, expanding the application of ILISs to additional matrices such as urine, tissue, and spinal fluid presents exciting opportunities. Urine, in particular, is widely used in metabolomic analysis due to its accessibility and rich metabolite content (7).

Data from negative ionization mode was acquired in this study, but not further evaluated given the time frame. However, this data could be made available and investigated to enhance the findings. Moreover, future research should adopt a more comprehensive approach, integrating the ILIS solution into broader clinical studies to observe its behavior. This will not only validate the findings of this study but also demonstrate the practical utility of incorporating ILIS into global metabolomic analyses, thus advancing its application in clinical diagnostics.

The ILISs made for this study also contained lipid ILISs as lipid analysis represents a crucial aspect of metabolomics. Lipidomic analysis was although not extensively covered in this study due to time constraints. Future work should incorporate lipidomics analysis using ILISs to enhance coverage and understanding of lipid metabolism in various biological samples.

Lastly, exploring quantification of metabolites using ILIS is an intriguing avenue for future research. Addressing the challenge posed by the vast number of metabolites will require careful method development. However, successful quantification could have significant implications for clinical diagnostics.

References

Cover image credit: The Metabolomics Innovation Centre https://metabolomicscentre.ca/

1. Rakel RE. diagnosis [Internet]. Britannica; 2024 [updated 2024 Jan 11; cited 2024 Jan 23]. Available from: https://www.britannica.com/science/diagnosis

2. Institute NC. diagnostic test USA: National Cancer Institute; [cited 2024 March 31]. Available from: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/diagnostic-test.

3. Beger RD, Dunn W, Schmidt MA, Gross SS, Kirwan JA, Cascante M, et al. Metabolomics enables precision medicine: "A White Paper, Community Perspective". Official journal of the Metabolomic Society. 2016;12(10):149.

4. McAlister FA, Laupacis A, Armstrong PW. Finding the right balance between precision medicine and personalized care. CMAJ : Canadian Medical Association journal = journal de l'Association medicale canadienne. 2017;189(33):E1065–E8.

5. Hasanzad M, Sarhangi N, Chimeh SE, Ayati N, Afzali M, Khatami F, et al. Precision medicine journey through omics approach. ournal of diabetes and metabolic disorders. 2022;21(1):881-8.

6. Sussulini A, editor. Metabolomics: From Fundamentals to Clinical Applications. Campinas: Springer International Publishing 2017.

7. Kiseleva O, Kurbatov I, Ilgisonis E, Poverennaya E. Defining Blood Plasma and Serum Metabolome by GC-MS. Metabolites. 2022;12(1):15.

8. Surendran A, Atefi N, Zhang H, Aliani M, Ravandi A. Defining Acute Coronary Syndrome through Metabolomics. Metabolites. 2021;11(685).

9. Imperlini E, Santorelli L, Orrù S, Scolamiero E, Ruoppolo M, Caterino M. Mass Spectrometry-Based Metabolomic and Proteomic Strategies in Organic Acidemias. BioMed research international. 2016:9210408.

10. Institute NC. metabolite USA: National Cancer Institute; [cited 2024 April 1]. Available from: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/metabolite.

11. database Thm. About the Human Metabolome Database: The human metabolome database; [cited 2024 May 16]. Available from: https://hmdb.ca/about.

12. Hansen SH, Pedersen-Bjergaard S. Bioanalysis of pharmaceuticals United Kingdom: Wiley & Sons; 2015 2015.

13. Stringer KA, McKay RT, Karnovsky A, Quémerais B, Lacy P. Metabolomics and Its Application to Acute Lung Diseases. Frontiers in immunology. 2016;7(44).

14. Wishart DS. Advances in metabolite identification. Bioanalysis. 2011;3(15):1769–82.

15. Lundanes E, Reubsaet L, Greibrokk T. Chromatography Basic Principles, Sample Preparations and Related Methods. Weinheim, Germany: Wiley-VCH; 2014.

16. Harris DC. Quantitative Chemical Analysis. Eight ed. New York: W. H. Freeman and Company; 2010.

17. Zhou B, Xiao JF, Tuli L, Ressom HW. LC-MS-based metabolomics. Molecular bioSystems. 2012;8(2):470–81.

18. Fisher T. Untargeted Metabolomics Workflows: Thermo Fisher; 2006-2024 [cited 2024 April 16]. Available from: https://www.thermofisher.com/no/en/home/industrial/mass-spectrometry/mass-spectrometry-learning-center/mass-spectrometry-applications-area/metabolomics-mass-spectrometry/metabolomics-workflows/untargeted-metabolomics-workflows.html.

19. Fisher T. Compound Discoverer Software: Thermo Fisher; 2006-2024 [cited 2024 April 26]. Available from: https://www.thermofisher.com/no/en/home/industrial/mass-spectrometry/liquid-chromatography-mass-spectrometry-lc-ms/lc-ms-software/multi-omics-data-analysis/compound-discoverer-software.html.

20. Fenaille F, Saint-Hilaire PB, Rousseau K, Junot C. Data acquisition workflows in liquid chromatography coupled to high resolution mass spectrometry-based metabolomics: Where do we stand? Journal of chromatography. 2017;1526:1-12.

21. Broadhurst D, Goodacre R, Reinke SN, Kuligowski J, Wilson ID, Lewis MR, Dunn WB. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. Metabolomics : Official journal of the Metabolomic Society. 2018;14(6):72.

22. Broeckling CD, Richard D Beger, Cheng LL, Cumeras R, Cuthbertson DJ, Dasari S, et al. Current Practices in LC-MS Untargeted Metabolomics: A Scoping Review on the Use of Pooled Quality Control Samples. Analytical chemistry. 2023;95(51):18645–54.

23. Fisher T. Metabolomics Data Analysis 2006-2024 [cited 2024 April 28]. Available from: https://www.thermofisher.com/no/en/home/industrial/mass-spectrometry/mass-spectrometry-learning-center/mass-spectrometry-applications-area/metabolomics-mass-spectrometry/metabolomics-data-analysis.html.

24. Skoog DA, West DM, Holler FJ, Crouch SR. Fundamentals of analytical chemistry. Tenth Edition ed. USA: Cengage; 2021.

25. Zvelebil M, Baum JO. Understanding Bioinformatics. USA: Garland Science, Taylor & Francis Group, LLC, an informa business; 2008.

26. Schrimpe-Rutledge AC, Codreanu SG, Sherrod SD, McLean JA. Untargeted metabolomics strategies – Challenges and Emerging Directions. Journal of the American Society for Mass Spectrometry. 2016;27(12):1897–905.

27. Bueschl C, Kluger B, Lemmens M, Adam G, Wiesenberger G, Maschietto V, et al. A novel stable isotope labelling assisted workflow for improved untargeted LC–HRMS based metabolomics research. Metabolomics : Official journal of the Metabolomic Society. 2014;10(4):754–69.

28. Zhou W, Yang S, Wang PG. Matrix effects and application of matrix effect factor. Bioanalysis. 2017;9(23):1839–44.

29. Amer B, Deshpande RR, Souza A, Bird SS, Scientific TF, San Jose C. Quality assurance and quality control in metabolomics: achieving high-quality data for high-quality results USA: Thermo Fisher Scientific; 2023 [cited 2024 April 27]. Available from: https://assets.thermofisher.com/TFS-Assets/CMD/Technical-Notes/tn-001771-ov-pierce-amino-acid-standard-h-metabolomics-qaqc-tn001771-na-en.pdf.

30. Chokkathukalam A, Kim D-H, Barrett MP, Breitling R, Creek DJ. Stable isotope-labeling studies in metabolomics: new insights into structure and dynamics of metabolic networks. Bioanalysis. 2014;6(4):511–24.

31. Fu Y, Barkley D, Li W, Picard F, Flarakos J. Evaluation, identification and impact assessment of abnormal internal standard response variability in regulated LC-MS bioanalysis. Bioanalysis. 2020;12(8):545–59.

32. González-Domínguez Á, Estanyol-Torres N, Brunius C, Landberg R, González-Domínguez R. QComics: Recommendations and Guidelines for Robust, Easily Implementable and Reportable Quality Control of Metabolomics Data. Analytical chemistry. 2024;96(3):1064–72.

33. Livera AMD, Sysi-Aho M, Jacob L, ohann A. Gagnon-Bartsch, Castillo S, Simpson JA, Speed TP. Statistical methods for handling unwanted variation in metabolomics data. Analytical chemistry. 2016;87(7):3606–15.

34. Lippa KA, Aristizabal-Henao JJ, Beger RD, Bowden JA, Broeckling C, Beecher C, et al. Reference materials for MS-based untargeted metabolomics and lipidomics: a review by the metabolomics quality assurance and quality control consortium (mQACC). Metabolomics : Official journal of the Metabolomic Society. 2022;18(4):24.

35. Skogvold HB, Sandås EM, Østeby A, Løkken C, Rootwelt H, Rønning PO, et al. Bridging the Polar and Hydrophobic Metabolome in Single-Run Untargeted Liquid Chromatography-Mass Spectrometry Dried Blood Spot Metabolomics for Clinical Purposes. Journal of proteome research. 2021;20(8):4010–21.

36. Lipids AP. 330731 | EquiSPLASH® USA: Avanti Polar Lipids; 2024 [cited 2024 May 18]. Available from: https://avantilipids.com/product/330731.

37. Tiwari G, Tiwari R. Bioanalytical method validation: An updated review. Pharmaceutical methods. 2010;1(1):25-38.

Appendices

1. Instrumentation Settings for Liquid Chromatography-Mass Spectrometry Analysis
2. Analysis Results for Isotope-labeled Internal Standard Solution Optimization in EDTA Plasma
3. Concentrations of Metabolites in Patient Samples

Appendix 1: Instrumentation Settings for Liquid Chromatography-Mass Spectrometry Analysis

The liquid chromatography, mass spectrometric and electrospray system settings used in this study are presented in Tables 1-4 and Figure 1.

Table 1 System parameters for optimized LC-settings.

Parameters	Settings
Mobile phase A	Water + 0.1 % formic acid
Mobile phase B	Methanol + 0.1 % formic acid
Gradient	See Table 2 and Figure 1
Injection volume	2 µL
Column temperature	30 °C
Flow rate	300 µL/min
Analysis time	27.5 minutes
Re-equilibration time	10 minutes

Table 2 Flow gradient.

Time	% B
0	2
6	10
8.5	75
25	100
27.5	100

Note: The mobile phase consists of MP A and B from Table 1.

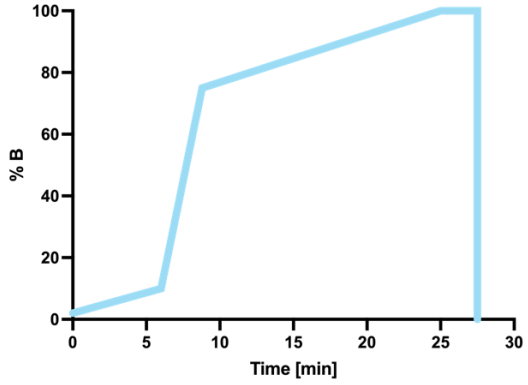


Figure 1 *Flow gradient with the same mobile phase as in Table 2.*

Table 3 System parameter setting for optimized MS-settings.

Parameters	Settings
Scan type	Full MS
Scan ranges	50-750 and 750-1700
Fragmentation	None
Resolution at m/z 200	70,000
Polarity	Positive and negative
Micro scans	1
Lock masses	Off
Automatic gain control target value	1.00E+06
Maximum injection time	250 milli seconds
Analysis time	27.5 minutes
Re-equilibrium time	10 minutes

Table 4 Parameter settings for the electrospray

Parameters	Settings
Sheath gas (N_2) flow rate	40 a.u.
Auxiliary gas (N_2) flow rate	10 a.u.
Sweep gas (N_2) flow rate	2 a.u.
Capillary temperature	250 ^oC
S-lens RF level	50.0
Auxiliary gas heater temperature	300 ^oC
Electrospray voltage	3.5 kV
Electrospray needle position	C
Capillary temperature	250 ^oC

Appendix 2: Analysis Results for Isotope-labeled Internal Standard Solution Optimization in EDTA Plasma

Table 1 presents the analysis results obtained during the optimization of the isotope-labeled internal standard (ILIS) solution concentrations in EDTA plasma.

Table 1 Analysis results for isotope-labeled internal standard solution optimization in EDTA plasma. Data represents the mean of six injections per concentration. Not detected (N.D.).

ILIS in EDTA plasma	Adduct	Concentration in stock solution [µM]	Concentration in HPLC vial [µM]	Area		RT	
				Mean [a.u.]	CV (%)	Mean [min]	CV (%)
Alanine (d4)	$[M+H]^+$	5,00	3,75	1,51E+07	3E+00	2,12	3E-01
		0,50	0,375	1,39E+06	1E+00	2,12	3E-01
		0,05	0,0375	1,16E+05	7E+00	2,12	4E-01
		0,005	0,00375	N.D	N.D.	N.D.	N.D.
Tryptophan (d5)	$[M+H]^+$	5,00	3,75	1,10E+08	1E+00	11,14	4E-02
		0,50	0,375	1,04E+07	3E+00	11,14	4E-02
		0,05	0,0375	1,05E+06	4E+00	11,14	4E-02
		0,005	0,00375	7,74E+04	3E+01	11,15	4E-02
Acylcarnitine C18 (d3)	$[M+H]^+$	5,00	3,75	5,42E+08	1E+00	14,91	1E-01
		0,50	0,375	4,86E+07	4E+00	14,91	7E-02
		0,05	0,0375	5,58E+06	3E+00	14,92	9E-02
		0,005	0,00375	6,21E+05	4E+01	14,94	1E-01
Uracil ^{15}N2	$[M+H]^+$	5,00	3,75	2,42E+07	2E+00	3,86	3E-01
		0,50	0,375	2,35E+06	3E+00	3,86	3E-01
		0,05	0,0375	1,57E+05	1E+01	3,86	7E-01
		0,005	0,00375	N.D.	N.D.	N.D.	N.D.

Table 2 presents the analysis results for pure isotope-labeled internal standards of different dilutions. The data in this table does not include any biological matrix.

Table 2 *Analysis results of isotope-labeled internal standard of different dilutions.*

Concentration	5,0		0,5		0,05		0,005	
ILIS	**Peak area [a.u.]**	**RT [min]**	**Peak area [a.u.]**	**RT [min]**	**Peak area [a.u.]**	**RT [min]**	**Peak area [a.u.]**	**RT [min]**
Alanine-d4	5.49E+07	2.11	5.59E+06	2.10	5.68E+05	2.10	3.98E+04	2.12
Tryptophan (d5)	1.68E+08	11.13	1.59E+07	11.13	1.73E+06	11.14	3.31E+04	11.16
Acylcarnitine C18 (d3)	8.66E+08	14.95	6.76E+07	14.95	7.13E+06	14.93	5.29E+05	14.93
Uracil 15N2	4.77E+07	3.85	5.04E+06	3.84	5.55E+05	3.85	1.04E+04	3.80

Appendix 3: Concentrations of Metabolites in Patient Samples

The concentration of alanine, tryptophan, acylcarnitine C18:0 and uracil in patient samples of EDTA plasma, heparin plasma and serum taken from the Oslo University Hospital diagnostic biobank (REK approval 173346). Note that heparin samples B – E are the same patient as serum samples B-E. Apart from this, the sample ID's are different patients.

Table 1 Concentrations of the EDTA plasma patient samples obtained by targeted analysis from the Oslo University Hospital diagnostic biobank.

Sample ID	Concentration (µmol/L)			
	Ala	Trp	C18-AC	U
A	265	29	0.041	not measured*
B	272	38	0.028	not measured*
C	198	21	0.052	not measured*
D	313	96	not measured*	not measured*
E	335	54	not measured*	not measured*

Note: Not all metabolites are measured for all the samples in the hospital routine lab as analyses are tailored based on the patient´s clinical needs and symptoms specified in the doctor's requisition.

Table 2 Concentrations of the heparin plasma patient samples obtained by targeted analysis from the Oslo University Hospital diagnostic biobank.

Sample ID	Concentration (µmol/L)			
	Ala	Trp	C18-AC	U
A_H	163	32	0.055	not measured*
B_H	175	83	not measured*	not measured*
C_H	432	36	not measured*	not measured*
D_H	335	47	not measured*	not measured*
E_H	508	33	not measured*	not measured*

Note: Not all metabolites are measured for all the samples in the hospital routine lab as analyses are tailored based on the patient´s clinical needs and symptoms specified in the doctor's requisition.

Table 3 Concentrations of the serum patient samples obtained by targeted analysis from the Oslo University Hospital diagnostic biobank.

Sample ID	Concentration (µmol/L)			
	Ala	Trp	C18-AC	U
A_S	231	40	not measured*	not measured*
B_S	175	83	not measured*	not measured*
C_S	432	36	not measured*	not measured*
D_S	not measured*	not measured*	0.042	not measured*
E_S	not measured*	not measured*	0.029	not measured*

Note: Not all metabolites are measured for all the samples in the hospital routine lab as analyses are tailored based on the patient´s clinical needs and symptoms specified in the doctor's requisition.